



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07H 21/04, C12N 15/63, 15/85, 15/08, 15/09, 15/00, A61K 48/00		A1	(11) International Publication Number: WO 00/14103 (43) International Publication Date: 16 March 2000 (16.03.00)
<p>(21) International Application Number: PCT/US99/18865</p> <p>(22) International Filing Date: 18 August 1999 (18.08.99)</p> <p>(30) Priority Data: 09/145,916 2 September 1998 (02.09.98) US</p> <p>(71) Applicant: BETH ISRAEL DEACONESS MEDICAL CENTER (US/US); 330 Brookline Avenue, Boston, MA 02215 (US).</p> <p>(72) Inventors: SIMONS, Michael, 115 Grove Street, Chestnut Hill, MA 02167 (US). VOLK, Rudiger, 8 Sussex Street, Boston, MA 02120 (US). HOROWITZ, Arie, 4503 Stearns Hill Road, Waltham, MA 02154 (US).</p> <p>(74) Agent: PRASHKER, David, 8 Chateau Heights, Magnolia, MA 01930 (US)</p>		<p>(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>With international search report Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments</i></p>	
<p>(54) Title: STIMULATION OF ANGIOGENESIS VIA ENHANCED ENDOTHELIAL EXPRESSION OF SYNDECAN-4 CORE PROTEINS</p> <p>(57) Abstract</p> <p>The present invention provides tangible means and methods for stimulation of angiogenesis via enhanced endothelial expression of core proteins having a syndecan-4 cytoplasmic region intracellularly. The tangible means include a prepared DNA sequence fragment having separate and individual DNA sequenced portions coding for an heparan sulfate binding extracellular domain, a central transmembrane domain, and a cytoplasmic domain coding for the syndecan-4 polypeptide. The prepared DNA sequence unitary fragment may be delivered to endothelial cells <i>in-situ</i>, both under <i>in-vivo</i> and/or <i>in-vitro</i> conditions, using suitable expression vectors including plasmids and viruses. The resulting transfected endothelial cells overexpress heparan sulfate binding, core proteins; and the resulting overexpression of these proteoglycan entities causes stimulation of angiogenesis <i>in-situ</i>.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
DY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

STIMULATION OF ANGIOGENESIS VIA ENHANCED ENDOTHELIAL
EXPRESSION OF SYNDECAN-4 CORE PROTEINS

5

FIELD OF THE INVENTION

The present invention is concerned generally with the stimulation of
10 angiogenesis in-situ in living tissues and organs; and is particularly directed to the preparation and use of prepared DNA sequences and expression vectors suitable for transfection of endothelial cells in-situ such that overexpression of extracellular matrix heparin sulfate binding proteoglycans subsequently occurs in-situ.

15

BACKGROUND OF THE INVENTION

Angiogenesis, by definition, is the formation of new capillaries and blood
20 vessels within living tissues; and is a complex process first recognized in studies of wound healing and then within investigations of experimental tumors. Angiogenesis is thus a dynamic process which involves extracellular matrix remodeling, endothelial cell migration and proliferation, and functional maturation of endothelial cells into mature blood vessels [Brier, G. and K. Alitalo, Trends
25 Cell Biology 6: 454-456 (1996)]. Clearly, in normal living subjects, the process of angiogenesis is a normal host response to injury, and as such, is an integral part of the host body's homeostatic mechanisms.

It will be noted and appreciated, however, that whereas angiogenesis represents an important component part of tissue response to ischemia, or tissue
30 wounding, or tumor-initiated neovascularization, relatively little new blood vessel formation or growth takes place in most living tissues and organs in mature adults (such as the myocardium of the living heart) [Folkman, J. and Y. Shing, J. Biol.

- 2 -

Chem. 267: 10931-10934 (1992); Folkman, J., Nat. Med. 1: 27-31 (1995); Ware, J.A. and M. Simons, Nature Med. 3: 158-164 (1997)]. Moreover, although regulation of an angiogenetic response in-vivo is a critical part of normal and pathological homeostasis, little is presently known about the control mechanisms for this process. A number of different growth factors and growth factor receptors have been found to be involved in the process of stimulation and maintenance of angiogenetic responses. In addition, a number of extracellular matrix components and cell membrane-associated proteins are thought to be involved in the control mechanisms of angiogenesis. Such proteins include SPARC [Sage et al., J. Cell Biol. 109: 341-356 (1989); Motamed, K. and E.H. Sage, Kidney Int. 51: 1383-1387 (1997)]; thrombospondin 1 and 2 respectively [Folkman, J., Nat. Med. 1: 27-31 (1995); Kyriakides et al., J. Cell Biol. 140: 419-430 (1998)]; and integrins $\alpha v\beta 5$ and $\alpha v\beta 3$ [Brooks et al., Science 264: 569-571 (1994); Friedlander et al., Science 270: 1500-1502 (1995)]. However, it is now recognized that a major role is played by heparan-binding growth factors such as basic fibrocyte growth factor (bFGF) and vascular endothelial growth factor (VEGF); and thus the means for potential regulation of angiogenesis involves the extracellular heparan sulfate matrix on the surface of endothelial cells.

Research investigations have shown that heparan sulfate core proteins or proteoglycans mediate both heparin-binding growth factor/receptor interaction at the cell surface; and that accumulation and storage of such growth factors within the extracellular matrix proper occurs [Vlodavsky et al., Clin. Exp. Metastasis 10: 65 (1992); Olwin, B.B. and A. Rapraeger, J. Cell Biol. 118: 631-639 (1992); Rapraeger, A.C., Curr. Opin. Cell Biol. 5: 844-853 (1993)]. The presence of heparin or heparan sulfate is required for bFGF-dependent activation of cell growth in-vitro [Yayon et al., Cell 64: 841-848 (1991); Rapraeger et al., Science 252: 1705-1708 (1991)]; and the removal of heparan sulfate chains from the cell surface and extracellular matrix by enzymatic digestion greatly impairs bFGF activity and inhibits neovascularization in-vivo [Sasisekharan et al., Proc. Natl. Acad. Sci. USA 91: 1524-1528 (1994)]. Ample scientific evidence now exists which demonstrates that any alteration of heparan sulfate (HS) chain composition

on the cell surface or within the extracellular matrix which is initiated by means of an altered synthesis, or a degradation, or a substantive modification of glycosaminoglycan (GAG) chains can meaningful affect the intracellular signaling cascade initiated by the growth factor. The importance of heparan sulfate in growth factor-dependent signaling has become well recognized and established in this field.

Heparan sulfate (HS) chains on the cell surface and within the extracellular matrix are present via binding to a specific category of proteins commonly referred to as "proteoglycans". This category is constituted of several classes of core proteins, each of which serve as acceptors for a different type of glycosaminoglycan (GAG) chains. The GAGs are linear co-polymers of N-acetyl-D-glycosamine [binding heparan sulfate] or N-acetyl-D-galactosamine [binding chondroitin sulfate (CS) chains] and acidic sugars which are attached to these core proteins via a linking tetrasaccharide moiety. Three major classes of HS-carrying core proteins are present in living endothelial cells: cell membrane-spanning syndecans, GPI-linked glypcans, and a secreted perlecan core protein [Rosenberg *et al.*, *J. Clin. Invest.* **99**: 2062-2070 (1997)]. While the perlecan and glypcan classes carry and bear HS chains almost exclusively, the syndecan core proteins are capable of carrying both HS and CS chains extracellularly. The appearance of specific glycosaminoglycan chains (such as HS and/or CS) extracellularly on protein cores is regulated both by the structure of the particular core protein as well as via the function of the Golgi apparatus intracellularly in a cell-type specific manner [Shworak *et al.*, *J. Biol. Chem.* **269**: 21204-21214 (1994)].

25 The syndecan class is composed of four closely related family proteins (syndecan-1,-2,-3 and -4 respectively) coded for by four different genes in-vivo. Syndecans-1 and -4 are the most widely studied members of this class and show expression in a variety of different cell types including epithelial, endothelial, and vascular smooth muscle cells, although expression in quiescent tissues is at a fairly low level [Bernfield *et al.*, *Annu. Rev. Cell Biol.* 8: 365-393 (1992); Kim *et al.*, *Mol. Biol. Cell* 5: 797-805 (1994)]. Syndecan-2 (also known as fibroglycan) is expressed at high levels in cultured lung and skin fibroblasts,

- 4 -

although immunocytochemically this core protein is barely detectable in most adult tissues. However, syndecan-3 (also known as N-syndecan) demonstrates a much more limited pattern of expression, being largely restricted to peripheral nerves and central nervous system tissues (although high levels of expression are shown in the neonatal heart) [Carey *et al.*, *J. Cell Biol.* **117**: 191-201 (1992)].

5 All members of the syndecan class are capable of carrying both HS and CS chains extracellularly, although most of syndecan-associated biological effects (including regulation of blood coagulation, cell adhesion, and signal transduction) are largely thought to be due to the presence of HS chains capable of binding 10 growth factors, or cell adhesion receptors and other biologically active molecules [Rosenberg *et al.*, *J. Clin. Invest.* **99**: 2062-2070 (1997)].

Curiously, however, very little is presently known about and relatively little research attention has been paid to the function of the syndecan core proteins in-situ. Syndecan-1 expression has been observed during development suggesting 15 a potential role in the epithelial organization of the embryonic ectoderm and in differential axial patterning of the embryonic mesoderm, as well as in cell differentiation [Sutherland *et al.*, *Development* **113**: 339-351 (1991); Trautman *et al.*, *Development* **111**: 213-220 (1991)]. Also, mesenchymal cell growth during tooth organogenesis is associated with transient induction of syndecan-1 gene 20 expression [Vainio *et al.*, *Dev. Biol.* **147**: 322-333 (1991)]. Furthermore, in adult living tissues, expression of syndecan-1 and syndecan-4 proteoglycans increases within arterial smooth muscle cells after balloon catheter injury [Nikkari *et al.*, *Am. J. Pathol.* **144**: 1348-1356 (1994)]; in healing skin wounds [Gallo *et al.*, *Proc. Natl. Acad. Sci. USA* **91**: 11035-11039 (1994)]; and in the heart 25 following myocardial infarction [Li *et al.*, *Circ. Res.* **81**: 785-796 (1997)]. In the latter instances, the presence of blood-derived macrophages appears necessary for the induction of syndecan-1 and -4 gene expression. However, the effects of changes in syndecan expression on cell behavior are presently not well understood. For example, this core protein is believed to mediate bFGF binding 30 and cell activity. Overexpression of syndecan-1 in 3T3 cells led to inhibition of bFGF-induced growth [Mali *et al.*, *J. Biol. Chem.* **268**: 24215-24222 (1993)]; while in 293T cells, overexpression of syndecan-1 augmented serum-dependent

- 5 -

growth [Numa *et al.*, *Cancer Res.* **55**: 4676-4680 (1995)]. Furthermore, syndecan-1 overexpression showed increased inter-cellular adhesion in lymphoid cells [Lebakken *et al.*, *J. Cell Biol.* **132**: 1209-1221 (1996)] while also decreasing the ability of B-lymphocytes to invade collagen gels [Liberbach, B.F. and R.D. Sanderson, *J. Biol. Chem.* **269**: 20013-20019 (1994)]. These ostensibly contradictory findings have merely added to the uncertainty and the disparity of knowledge regarding the effects of syndecan expression.

In comparison, the glycan core protein class is composed of five murine and human members and a *Drosophila dally* homologue [Rosenberg *et al.*, *J. Clin. Invest.* **99**: 2062-2070 (1997)]. Unlike syndecans, the glycan members are fully extracellular proteins attached to the cell membrane via a GPI anchor. Only one member of the class, glycan-1, is expressed in endothelial cells. Another unique feature of the glycan class of proteoglycans is that they carry substantially only heparan sulfate (HS) chains [Aviezer *et al.*, *J. Biol. Chem.* **269**: 114-121 (1994)]. Consequently, while little is presently known about the biological function of glycans, they appear able to stimulate FGF receptor 1 occupancy by bFGF and appear able to promote biological activity for several different FGF family members [Steinfeld *et al.*, *J. Cell Biol.* **133**: 405-416 (1996)].

Finally, perlecan is the third and last class of heparan sulfate (HS)-carrying core proteins. Perlecan is a secreted proteoglycan that also has been implicated in regulation of bFGF activity [Aviezer *et al.*, *Mol. Cell Biol.* **17**: 1938-1946 (1997); Steinfeld *et al.*, *J. Cell Biol.* **133**: 405-416 (1996)]. However, little is known regarding this basal lamina proteoglycan beyond its interaction with basic fibroblast growth factor receptor.

In sum therefore, it is evident that the quantity and quality of knowledge presently available regarding glycosaminoglycan (GAG) binding core proteins is factually incomplete, often presumptive, and in some instance apparently contradictory. Clearly the role of specific proteoglycans as mediators under varying conditions is recognized; nevertheless, the mechanisms of action and the functional activity of the various individual classes of core proteins yet remains to be elucidated in full. Thus, while the role of proteoglycans in some manner

- 6 -

relates to angiogenesis, there is no evidence or data known to date which clearly establishes the true functional value of proteoglycans nor which establishes a use for proteoglycans as a means for stimulating angiogenesis in-situ.

5

SUMMARY OF THE INVENTION

10 The present invention has multiple aspects and is definable in multiple contexts. A first primary aspect and definition provides a prepared DNA segment for placement in a suitable expression vector and transfection of endothelial cells in-situ such that overexpression of extracellular matrix heparan sulfate proteoglycan entities subsequently occurs in-situ, said prepared DNA segment comprising:

15 at least one first DNA sequence coding for the extracellular domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said extracellular domain first DNA sequence specifying the extracellular N-terminal portion of an expressed proteoglycan entity which is then located at and extends from the endothelial cell surface and is capable of binding heparan sulfates to form an extracellular matrix in-situ.

20 at least one second DNA sequence coding for the transmembrane domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said transmembrane domain second DNA sequence specifying the medial portion of an expressed proteoglycan entity which is then located at and extends through the endothelial cell membrane and is joined with said extracellular N-terminal portion of said expressed proteoglycan entity; and

25 at least one third DNA sequence coding for the cytoplasmic domain of the syndecan-4 molecule in said discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said syndecan-4 cytoplasmic domain third DNA sequence specifying the cytoplasmic portion of an expressed proteoglycan entity which is then present within the cytoplasm of a transfected endothelial cell and is joined to said transmembrane portion and said extracellular N-terminal portion of said expressed proteoglycan entity.

- 7 -

A second primary aspect and definition provides a constructed expression vector for transfection of endothelial cells in-situ such that overexpression of extracellular matrix heparan sulfate proteoglycan entities subsequently occurs in-situ, said constructed expression vector comprising:

5 a prepared DNA segment comprised of

 (i) at least one first DNA sequence coding for the extracellular domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said extracellular domain first DNA sequence specifying the extracellular N-terminal portion of an expressed proteoglycan entity which is then located at and extends from the endothelial cell surface and is capable of

10 binding heparan sulfates to form an extracellular matrix in-situ,

 (ii) at least one second DNA sequence coding for the transmembrane domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said transmembrane domain second DNA sequence specifying the medial portion of an expressed proteoglycan entity which is then located at and extends through the endothelial cell membrane and is joined with said extracellular N-terminal portion of said expressed proteoglycan entity, and

15 (iii) at least one third DNA sequence coding for the cytoplasmic domain of the syndecan-4 molecule in said discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said syndecan-4 cytoplasmic domain third DNA sequence specifying the cytoplasmic portion of an expressed proteoglycan entity which is then present within the cytoplasm of a transfected endothelial cell and is joined to said transmembrane portion and said extracellular N-terminal portion of said expressed proteoglycan entity; and

20 an expression vector carrying said prepared DNA segment and suitable for transfection of endothelial cells in-situ.

30 A third primary aspect and definition provides an in-situ transfected endothelial cell which overexpresses extracellular matrix heparan sulfate proteoglycans and positions on the proteoglycans at the cell surface, said in-situ transfected endothelial cell comprising:

a viable endothelial cell previously transfected in-situ with a constructed expression vector such that said transfected endothelial cell overexpresses discrete extracellular matrix heparan sulfate proteoglycan entities coded for by said vector, said overexpressed proteoglycan entities being comprised of

5 (i) an extracellular N-terminal portion which is located at and extends from the transfected endothelial cell surface and which binds heparan sulfates to form an extracellular matrix in-situ, said extracellular N-terminal portion being the expressed product of at least one first DNA sequence in the constructed expression vector coding for the extracellular domain of said proteoglycan entity expressed by the transfected endothelial cell in-situ,

10 (ii) a transmembrane medial portion which is located at and extends through the endothelial cell membrane and is joined with said extracellular N-terminal portion of said proteoglycan entity, said transmembrane medial portion being the expressed product of at least one second DNA sequence in the constructed expression vector coding for the transmembrane domain of said proteoglycan entity expressed by the transfected endothelial cell in-situ, and

15 (iii) a syndecan-4 cytoplasmic portion present within the cytoplasm of the transfected endothelial cell which is joined to said transmembrane portion and said extracellular N-terminal portion of said proteoglycan entity, said syndecan-4 cytoplasmic portion being the expressed product of at least one third DNA sequence in the constructed expression vector coding for the cytoplasmic domain of the syndecan-4 molecule of said proteoglycan entity expressed by the transfected endothelial cell in-situ.

25 A fourth primary aspect and definition provides a method for stimulating angiogenesis in-situ within a living tissue comprising vascular endothelial cells, said method comprising the steps of:

20 transfecting vascular endothelial cells within a living tissue with a constructed expression vector such that the resulting transfected vascular endothelial cells overexpress discrete extracellular matrix heparan sulfate proteoglycan entities coded for by said constructed expression vector, said overexpressed proteoglycan entities being comprised of

- 9 -

(i) an extracellular N-terminal portion which is located at and extends from the transfected vascular endothelial cell surface and binds heparan sulfates to form an extracellular matrix in-situ, said extracellular N-terminal portion being the expressed product of at least one first DNA sequence in the 5 constructed expression vector coding for the extracellular domain of said proteoglycan entity expressed by a transfected vascular endothelial cell in-situ,

(ii) a transmembrane medial portion which is located at and extends through a transfected vascular endothelial cell membrane and is joined with said extracellular N-terminal portion of said proteoglycan entity, said 10 transmembrane medial portion being the expressed product of at least one second DNA sequence in the constructed expression vector coding for the transmembrane domain of said proteoglycan entity expressed by a transfected vascular endothelial cell in-situ, and

(iii) a syndecan-4 cytoplasmic portion present within the 15 cytoplasm of a transfected vascular endothelial cell which is joined to said transmembrane portion and said extracellular N-terminal portion of said expressed proteoglycan entity, said syndecan-4 cytoplasmic portion being the expressed product of at least one third DNA sequence in the constructed expression vector coding for the cytoplasmic domain of the syndecan-4 molecule of said 20 proteoglycan entity expressed by a transfected vascular endothelial cell in-situ; and

allowing said transfected vascular endothelial cells bearing said overexpressed extracellular matrix proteoglycan entities to stimulate angiogenesis in-situ.

25

BRIEF DESCRIPTION OF THE FIGURES

The present invention can be more easily understood and better 30 appreciated when taken in conjunction with the accompanying drawing, in which:
Fig. 1 is a representation of a prepared DNA sequence fragment;

- 10 -

Fig. 2 is a recitation of the DNA sequence coding for the extracellular domain of syndecan-1;

Fig. 3 is a recitation of the DNA sequence coding for extracellular domain of syndecan-2;

5 Fig. 4 is a recitation of the DNA sequence coding for the extracellular domain of syndecan-3;

Fig. 5 is a recitation of the DNA sequence coding for the extracellular domain of syndecan-4;

10 Fig. 6 is a recitation of the DNA sequence coding for the extracellular domain of glypcan-1;

Fig. 7 is a recitation of the DNA sequence coding for the transmembrane domain of syndecan-1;

Fig. 8 is a recitation of the DNA sequence coding for the transmembrane domain of syndecan-2;

15 Fig. 9 is a recitation of the DNA sequence coding for the transmembrane domain of syndecan-3;

Fig. 10 is a recitation of the DNA sequence coding for the transmembrane domain of syndecan-4;

20 Fig. 11 is a recitation of the DNA sequence coding for the transmembrane domain of GPI;

Fig. 12 is a recitation of the DNA sequence coding for the transmembrane domain of perlecan;

Fig. 13 is a recitation of the DNA sequence coding for the cytoplasmic domain of syndecan-4;

25 Fig. 14 is a graph illustrating the in-vitro growth assays of ECV-derived cell clones;

Figs. 15A-15C are photographs showing the results of Matrigel growths assays;

30 Fig. 16 is a graph illustrating the effect of syndecan construct expression on endothelial cell migration in Boyden chamber assays;

Figs. 17A-17F are photographs showing BudR uptake in op/op homozygous (-/-) and heterozygous (+/-) mice;

- 11 -

Fig. 18 is a photograph showing Northern blot analysis of gene expression in PR-39 transgenic mice; and

Fig. 19 is a graph illustrating in-vitro microvascular reactivity in PR-39 transgenic mice.

5

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides both the tangible means and the methods 10 for causing an overexpression of extracellular, heparan sulfate carrying, proteoglycans on-demand at and through the surface of endothelial cells; and via such on-demand overexpression of proteoglycans to stimulate angiogenesis in-situ. The tangible means include a prepared DNA segment comprising sequences 15 coding for an extracellular domain, a transmembrane domain, and the cytoplasmic domain of the syndecan-4 protein; as well as a constructed expression vector for the transfection of endothelial cells in-situ such that overexpression of extracellular matrix, heparan sulfate bearing, proteoglycan entities subsequently occurs in-situ. The resulting transfected endothelial cell overexpresses 20 proteoglycans and positions them at the cell surface - thereby providing the structural and functional entities by which to stimulate angiogenesis in-situ.

A number of major benefits and advantages are therefore provided by the means and methods comprising the present invention. These include the following:

1. The present invention provides in-situ stimulation for angiogenesis. By 25 definition, therefore, both in-vivo and in-vitro circumstances of use and application are envisioned and expected. Moreover, the endothelial cells which are to be transfected such that overexpression of proteoglycans subsequently occurs, may be alternatively isolated endothelial cells, be part of living tissues comprising a variety of other cells such as fibrocytes and muscle cells, and may 30 also comprise part of specific organs in the body of a living human or animal subject. While the user shall choose the specific conditions and circumstances for practicing the present invention, the intended scope of application and the

- 12 -

envisioned utility of the means and methods described herein apply broadly to living cells, living tissues, functional organs and systems, as well as the complete living body unit as a viable whole.

2. The present invention has a variety of different applications and uses. Of 5 clinical and medical interest and value, the present invention provides the opportunity to stimulate angiogenesis in tissues and organs in a living subject which has suffered defects or has undergone anoxia or infarction. A common clinical instance is the myocardial infarction or chronic myocardial ischemia of heart tissue in various zones or areas of a living human subject. The present 10 invention thus provides opportunity and means for specific site stimulation and inducement of angiogenesis under controlled conditions. The present invention also has major research value for research investigators in furthering the quality and quantity of knowledge regarding the mechanisms controlling angiogenesis under a variety of different conditions and circumstances.

15 3. The present invention envisions and permits a diverse range of routes of administration and delivery means for introducing a constructed expression vector to a specific location, site, tissue, organ, or system in the living body. A variety of different expression vectors are available to the practitioner; and a diverse and useful range of delivery systems which are conventionally available and in 20 accordance with good medical practice are adapted directly for use. In this manner, not only are the means for transfection under the control of the user, but also the manner of application and limiting the locale or area of intentional transfection of endothelial cells can be chosen and controlled.

25 4. The user also has the choice and discretion of the manner in which the DNA segment is prepared - so long as the prepared DNA fragment conforms to the minimal requirements set forth herein. Thus, the prepared DNA sequence fragment may comprise the entire syndecan-4 DNA sequence in each of the required extracellular, transmembrane, and cytoplasmic domains. However, it is expected and envisioned that the more frequent choice will be a chimera core 30 protein structure which comprises only the syndecan-4 cytoplasmic domain but incorporates transmembrane and extracellular domains which are not native to the DNA of syndecan-4. Thus, the majority of prepared DNA sequenced fragments

- 13 -

will be chimeric DNA segments ligated together intentionally using recombinant techniques and methods to form a unitary DNA fragment.

5. The present invention provides a unique capability and control for stimulating angiogenesis in-situ by genetic manipulation of the endothelial cells as they exist within the tissues and organs as found. This level of gene control and utilization of the expression mechanisms found within the cytoplasms of the endothelial cells themselves provides a point of intentional intervention which harnesses and utilizes the cellular systems of the endothelial cells themselves to produce the intended and desired result. The transfected endothelial cells in-situ are thus minimally altered from their original genetic constituents; and the methodology utilizes the natural regulatory and protein producing systems of the endothelial cells themselves to provide the overexpression of proteoglycans which are located and positioned in the normally expected manner by the endothelial cells as part of the normal homeostatic mechanisms.

15 Accordingly, by the very requirements of the present invention it is thus important, if not essential, that the user be at least familiar with the many techniques for manipulating and modifying nucleotides and DNA fragments which have been reported and are today widespread in use and application. Merely exemplifying the many authoritative texts and published articles presently 20 available in the literature regarding genes, DNA nucleotide manipulation and the expression of proteins from manipulated DNA fragments are the following: Gene Probes for Bacteria (Macario and De Marcario, editors) Academic Press Inc., 1990; Genetic Analysis, Principles Scope and Objectives by John R.S. Ficham, Blackwell Science Ltd., 1994; Recombinant DNA Methodology II (Ray Wu, 25 editor), Academic Press, 1995; Molecular Cloning, A Laboratory Manual (Maniatis, Fritsch, and Sambrook, editors), Cold Spring Harbor Laboratory, 1982; PCR (Polymerase Chain Reaction), (Newton and Graham, editors), Bios Scientific Publishers, 1994; and the many references individually cited within 30 each of these publications. All of these published texts are expressly incorporated by reference herein.

In addition, a number of issued U.S. Patents and published patent applications have been issued which describe much of the underlying DNA

- 14 -

technology and many of the conventional recombinant practices and techniques for preparing DNA sequences coding for core proteins such as syndecan-4. Merely exemplifying some of the relevant patent literature for this subject are: U.S. Patent Nos. 5,486,599; 5,422,243; 5,654,273; 4,356,270; 4,331,901; 5 4,273,875; 4,304,863; 4,419,450; 4,362,867; 4,403,036; 4,363,877; as well as Publications Nos. W09534316-A1; W09412162-A1; W09305167-A1; W09012033-A1; W09500633; W09412162; and R09012033. All of these patent literature publications are also expressly incorporated by reference herein.

10

I. Constructed Core Protein DNA Fragments

A primary component part of the subject matter as a whole comprising the present invention is the manufacture and proper use of a prepared DNA segment intended for placement in a suitable expression vector; and useful for transfection 15 of endothelial cells *in-situ*, under both in-vivo and in-vitro conditions, such that overexpression of extracellular matrix heparan sulfate carrying proteoglycans subsequently occurs *in-situ*. The prepared DNA segment is a manufactured or 20 synthesized nucleotide fragment which preferably exists as a single, coiled strand of DNA bases in series; and constitutes sufficient DNA information to code for three requisite domains as illustrated by Fig. 1.

Fig. 1 is a simplistic and broadly representational illustration of the prepared DNA fragment after manufacture or synthesis. As seen therein, the prepared DNA segment comprises at least a first DNA sequence coding for the extracellular domain of a discrete and identifiable proteoglycan entity which, after 25 being expressed by a transfected endothelial cell *in-situ*, yields a specified N-terminal portion of an expressed proteoglycan entity. This N-terminal portion is the extracellular region of the expressed proteoglycan molecule which is then located at and extends from the transfected endothelial cell surface. This extended, extracellular N-terminal region (expressed as specific amino acid 30 residues in sequence) is capable of binding heparan sulfates at the cell surface thereby forming an extracellular heparan sulfate matrix *in-situ*.

- 15 -

The prepared DNA segment fragment illustrated by Fig. 1 must also provide at least one second DNA sequence coding for the transmembrane domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ. This transmembrane domain second DNA sequence codes for and 5 specifies the amino acid residue sequence of the medial or central portion of an expressed proteoglycan entity by the transfected endothelial cell. The medial portion or central region of the expressed proteoglycan is located at and extends through the endothelial cell membrane and is directly joined with and to the extracellular N-terminal portion of the expressed proteoglycan then extending 10 from the cell surface.

The final requisite component of the prepared DNA segment illustrated by Fig. 1 comprises at least one third DNA sequence coding for the cytoplasmic domain of the syndecan-4 molecule within the discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ. This third DNA sequence 15 specifies the cytoplasmic domain of the syndecan-4 DNA; and thus requires the expression of the particular amino acid residues which identify the syndecan-4 cytoplasmic region of the syndecan-4 core protein structure. While some small variation is permitted within the third DNA sequence specifying the cytoplasmic domain of the syndecan-4 amino acid structure, it is essential and required in 20 every embodiment of the prepared DNA fragment which is the present invention that the expressed cytoplasmic region of the proteoglycan entity then present within the cytoplasm of a transfected endothelial cell be identifiably recognized as being a syndecan-4 amino acid residue type. In addition, the expressed cytoplasmic portion constituting the syndecan-4 amino acid sequence must be 25 present within the cytoplasm of a transfected endothelial cell; and be joined to the transmembrane portion and the extracellular N-terminal portion of the expressed proteoglycan entity.

The heterogeneous domains joined together as a unitary fragment

30 It will be recognized and appreciated that the prepared DNA sequence is intended to be primarily, but not always, a heterogeneous DNA structure which joins together individual and separate DNA sequences as a unitary fragment. The

- 16 -

cytoplasmic domain constituting the third DNA sequence of the prepared fragment is limited and restricted to those DNA bases in sequence which recognizably and identifiably code for the syndecan-4 amino acid residues. Although single point or small variant alternations or modifications in the DNA 5 base sequence is permissible and expected, the overall domain must be in each and every instance recognizable and identifiable (using appropriate analytical means) as representative of the cytoplasmic region of the syndecan-4 molecular structure.

In comparison, the practitioner or intended user has the choice of many 10 different DNA sequences and formats when choosing and selecting DNA sequences coding for the extracellular domain coding for the N-terminal region and the transmembrane domain coding for the central or medial region of the proteoglycan molecule to be expressed. Thus, the user may construct the entirety 15 of the syndecan-4 DNA base sequence in its entirety such that a complete syndecan-4 core protein is subsequently expressed by a transfected endothelial cell. However, it is expected that in many instances the heterogeneous combination of individual and separate DNA base sequences representative of other and different core protein structures will be utilized; and that the resulting expressed proteoglycan entity will therefore be a chimeric core protein having 20 different amino acid residues constituting the transmembrane region and the extracellular region of the expressed proteoglycan entity. Thus it is expected and envisioned that the first DNA sequence may be the DNA coding for the glycan-1 amino acid residues; while the second DNA sequence coding for the transmembrane domain may be representative of the syndecan-1 amino acid 25 structure. Thus, the availability and use of heterogeneous prepared DNA fragments linking together first, second, and third DNA sequences - each of which is representative of a different core protein content and structure - thus will yield the expression of a chimeric proteoglycan entity which does not and cannot occur in nature.

30 In addition, the present availability of manufacturing heterogeneous DNA fragments which will yield an expressed chimera core protein in a transfected endothelial cell in-situ allows the intended user to choose and more carefully align

- 17 -

the amino acid composition of the expressed proteoglycan entity to be in accordance with and more compatible to the particular clinical problem and specific living tissue which is the intended treatment target. Thus, if damaged myocardium is the tissue intended as the target for treatment, the manufacture of 5 the heterogeneous fragment might include an extracellular domain (the first DNA sequence) coding for the glycan-1 region; which is joined to the transmembrane DNA domain (the second DNA sequence) which itself codes for a syndecan-2 amino acid region; which in turn is linked to the cytoplasmic domain (the third DNA sequence) which must code for the syndecan-4 region. In comparison, 10 however, if the targeted tissue is lung tissue, the extracellular domain might be representative of the syndecan-1 amino acid region; while the transmembrane domain represents the DNA coding for the amino acids of the syndecan-3 region; and the cytoplasmic domain continues to code exclusively for the syndecan-4 region. In other words, the extracellular domain can be specifically tailored to an 15 environment where it will be expressed.

In this manner, the manufacturer or intended user may customize and tailor the DNA sequences constituting the extracellular domain and/or the transmembrane domain as far as possible to best meet or suit the particular tissue, clinical condition, or pathology then existing and critical to the particular 20 application of interest. The range and variety of choices, therefore, allows the manufacturer and intended user a greater degree of flexibility, of potential therapeutic effects, and a greater degree of individuality than has ever been possible before the present invention was made.

25 Manufacture of the prepared DNA sequence fragment

It is expected and intended that the conventionally known and commonly used recombinant DNA materials, procedures, and instrumentation will be employed for the manufacture of the prepared DNA sequence fragments. Thus, the entire prepared DNA sequence structure including the entirety of the 30 extracellular domain and the transmembrane domain, and the cytoplasmic domain coding for the syndecan-4 structure may be synthesized directly from individual bases using the commercially available instruments and techniques. Alternatively,

- 18 -

the DNA sequences existing in naturally occurring core proteins may be replicated; and the cDNA isolated from individual clones using the appropriate enzymes and protocols. Regardless of the methods and means of manufacture, any and all of these protocols, procedures, systems, or instruments which will 5 yield the prepared DNA sequence as an discrete fragment is suitable and appropriate for use with the present invention.

A preferred technique, procedure, and methodology for preparing the DNA fragment as a whole is given in the Materials and Methods portion of the Experiments presented hereinafter. The described method, however, is merely 10 one among many conventionally known and available for this purpose.

A. The Extracellular Domain DNA Sequence

The manufacturer or user has a substantial choice in the range and variety of the DNA sequences suitable for use as the extracellular domain. A 15 representative, but non-exhaustive, listing of suitable choices is provided by Table 1 below.

- 19 -

Table 1: Representative Extracellular Domain DNA Sequence Fragments

	<u>Extracellular Domain</u> <u>Type Variant</u>	<u>DNA Sequence</u> <u>Recited By</u>
5	syndecan-1	Fig. 2
10	syndecan-2	Fig. 3
	syndecan-3	Fig. 4
15	syndecan 4	Fig. 5
	glypican-1	Fig. 6

- 20 -

B. The Transmembrane Domain DNA Sequences

The manufacturer or user also has substantial choice in the range and variety of the DNA sequences to be used as the transmembrane domain sequence coding for the medial or central region of the expressed proteoglycan entity. A representative, but non-exhaustive, listing of the second DNA sequence in the prepared fragment constituting and coding for the transmembrane domain is provided by Table 2 below.

- 21 -

Table 2: Representative Transmembrane Domain DNA Sequence Fragments

	Transmembrane Domain <u>Type</u> <u>Variant</u>	DNA Sequence <u>Recited By</u>
5	syndecan-1	Fig. 7
10	syndecan-2	Fig. 8
	syndecan-3	Fig. 9
	syndecan 4	Fig. 10
15	GPI	Fig. 11
	perlecan	Fig. 12

C. The Cytoplasmic Domain Coding For The Syndecan-4 Peptide

The third requisite cytoplasmic domain must code for the amino acid residue structure representative of the syndecan-4 core protein. As shown 5 experimentally by the data presented hereinafter, only the syndecan-4 cytoplasmic region and peptide structure allows for functional stimulation of angiogenesis in-situ. For this reason, it is essential and required in each embodiment of the present invention that the third DNA sequence coding for the cytoplasmic domain in the expressed proteoglycan entity in a transfected endothelial cell be 10 representative of and analytically identifiable as the syndecan-4 amino acid residue structure. A representative recitation of the DNA constituting the cytoplasmic domain of the syndecan-4 molecule is presented by Fig. 13 herein.

It will be noted and recognized that very little variability and substitution within the specific DNA base sequencing of the cytoplasmic domain of the 15 syndecan-4 molecule is permitted. While some changes are expected, be they point mutations, block substitutions and the like, the expected or envisioned degree of variability which might be present or permitted for the cytoplasmic domain DNA is believed to be quite limited.

As representative examples: The last four amino acids (EFYA) cannot be 20 changed or modified. Similarly, regarding the Serine residue at position 181: a mutation to an Alanine residue potentiates activation; while a mutation to Glutamate inhibits cell growth in a dominant fashion (dominant-negative mutation). Finally, the LGKKPIYKK sequences probably cannot be altered at all.

25

Expression Vectors And Means For Delivery In-Situ

A variety of methods are conventionally known and presently available to the user or practitioner of the present invention in order to introduce and deliver 30 a prepared DNA sequence fragment to the intended target in-situ. The means for delivery envision and include in-vivo circumstances; ex-vivo specimens and conditions; and in-vitro culture circumstances. In addition, the present invention intends and expects that the use of the prepared DNA sequence fragment in a

- 23 -

suitable expression vector and route of administration will be delivered to living tissues comprising endothelial cells, and typically vascular endothelial cells which constitute the basal layer of cells in capillaries and blood vessels generally. Clearly, the cells themselves are thus eukaryotic, typically mammalian cells from 5 human and animal origin; and most typically would include the higher order mammals such as humans and domesticated animals kept as pets or sources of food intended for consumption. Accordingly, the range of animals includes all domesticated varieties involved in nutrition including cattle, sheep, pigs and the like; as well as those animals typically used as pets or raised for commercial 10 purposes including horses, dogs, cats, and other living mammals typically living with and around humans.

Clearly, the expression vectors then must be suitable for transfection of endothelial cells in living tissues of mammalian origin and thus be compatible with that type and condition of cells under both in-vivo and/or in-vitro conditions. 15 The expression vectors thus typically include plasmids and viruses as expression vectors.

The range and variety of plasmids suitable for use with the present invention are broadly available and conventionally known in the technical and scientific literature. A representative, but non-exhaustive, listing is provided by 20 Table 3 below.

- 24 -

Table 3: Preferred Mammalian Plasmid Expression Vectors

	Plasmid Vectors
5	pH β -APr-1-neo EBO-pcD-XN pcDNAI/amp pcDNAI/neo pRc/CMV
10	pSV2gpt pSV2neo pSV2-dhfr pTk2 pRSV-neo
15	pMSG pSVT7 pKo-neo pHyg
20	

Alternatively, a wide and divergent variety of viral expression vectors suitable for insertion of the prepared DNA sequence fragment and subsequent transfection of endothelial cells in-situ is conventionally known and commonly available in this field. The particular choice of viral vector and the preparation of 5 the fully constructed expression vector incorporating the prepared DNA sequence fragment is clearly a matter of personal convenience and choice to the intended manufacturer or user; but should be selected with a eye towards the intended application and the nature of the tissues which are the intended target. A representative, but non-exhaustive, listing of preferred viral expression vectors 10 suitable for use as constructed vectors bearing the prepared DNA sequence fragment is provided by Table 4 below.

- 26 -

Table 4: Preferred Viral Expression Vectors

Bovine papilloma virus (BPV-1);

5

Epstein-Barr virus (phEBO; pREP- derived, and p205);

Retrovirus;

10 Adenovirus;

AAV (adeno-associated virus)

Lentivirus

15

Clearly, both the plasmid based vectors and the viral expression vectors constitute means and methods of delivery which are conventionally recognized today as "gene therapy" modes of delivery. However, this overall approach is not the only means and method of delivery available for the present invention.

5

Injection of recombinant proteins

10 Intracoronary delivery is accomplished using catheter-based deliveries of recombinant human protein dissolved in a suitable buffer (such as saline) which can be injected locally (i.e., by injecting into the myocardium through the vessel wall) in the coronary artery using a suitable local delivery catheter such as a 10mm InfusaSleeve catheter (Local Med, Palo Alto, CA) loaded over a 3.0mm x 20mm angioplasty balloon, delivered over a 0.014 inch angioplasty guidewire. Delivery was accomplished by first inflating the angioplasty balloon to 30 psi, and then delivering the protein through the local delivery catheter at 80 psi over 15 30 seconds (this can be modified to suit the delivery catheter).

Intracoronary bolus infusion can be accomplished by a manual injection of the protein through an Ultrafuse-X dual lumen catheter (SciMed, Minneapolis, MN) or another suitable device into proximal orifices of coronary arteries over 10 minutes.

20 Pericardial delivery is accomplished by instillation of the protein-containing solution into the pericardial sac. The pericardium is accessed either via a right atrial puncture, transthoracic puncture or via a direct surgical approach. Once the access is established, the material is infused into the pericardial cavity and the catheter is withdrawn. Alternatively, the delivery is 25 accomplished using slow-release polymers such as heparin-alginate or ethylene vinyl acetate (EVAc). In both cases, once the protein is integrated into the polymer, the desired amount of polymer is inserted under the epicardial fat or secured to the myocardial surface using, for example, sutures. In addition, polymer can be positioned along the adventitial surface of coronary vessels.

30 Intramyocardial delivery can be accomplished either under direct vision following thoracotomy or using thoracoscope or via a catheter. In either case, the protein containing solution is injected using a syringe or other suitable device

- 28 -

directly into the myocardium. Up to 2 cc of volume can be injected into any given spot and multiple locations (up to 30 injections) can be done in each patient. Catheter-based injections are carried out under fluoroscopic, ultrasound or Biosense NOGA guidance. In all cases after catheter introduction into the left 5 ventricle the desired area of the myocardium is injected using a catheter that allows for controlled local delivery of the material.

III. Examplary Applications And Preferred Routes Of Administration

A variety of approaches, routes of administration, and delivery methods 10 are available using the constructed expression vector comprising an inserted DNA sequence fragment coding for a proteoglycan entity. A majority of the approaches and routes of administration described hereinafter are medical applications and specific clinical approaches intended for use with human patients having specific medical problems and pathologies. It is expected that the reader 15 is familiar generally with the typical clinical human problem, pathology, and medical conditions described herein; and therefore will be able to follow and easily understand the nature of the intervention clinically using the present invention and the intended outcome and result of the clinical treatment - particularly as pertains to the stimulation of angiogenesis under in-vivo treatment 20 conditions. A representative listing of preferred approaches is given by Table 5 below.

- 29 -

Table 5:
Preferred Routes Of Administration

5 Catheter-based (intracoronary) injections and infusions;

 Direct myocardial injection
 (intramyocardial guided);

10 Direct myocardial injection
 (direct vision-epicardial-open chest or under thoroscope guidance);

 Local intravascular delivery;

15 Liposome-based delivery;

 Delivery in association with "homing" peptides.

Experimental and Empirical Data

To demonstrate the merits and value of the present invention, a series of planned experiments and empirical data are presented below. It will be expressly understood, however, that the experiments described and the results provided are merely the best evidence of the subject matter as a whole which is the invention; and that the empirical data, while limited in content, is only illustrative of the scope of the invention envisioned and claimed.

10 A. Materials and Methods:

Expression constructs and cell culture

Immortalized ECV304 cells (ATCC, Bethesda, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 15 heat-inactivated 10% fetal bovine serum (FBS, Gibco-BRL), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO₂. Full length coding region cDNAs for rat syndecan-4 and rat glypican-1 expression constructs were prepared in a retroviral vector MSCV2.2 by cloning a BamHI/HpaI fragment of rat syndecan-4 into cDNA into BgIII/HpaI fragment vector and 20 BamH1/EcoR1 fragment of rat glypican-1 into BgIII/Eco R1 sites of the same vector. Syndecan/glypican chimeras were created via PCR mutagenesis; cloned into the pCDNA3; sequenced; and shuttled into the MSCV2.2 vector. The syndecan-4-GPI (S4-GPI) construct was created by deleting the C-terminal end of rat syndecan-4 sequence starting with ²⁴⁷Gln and replacing it with the C-terminal 25 sequence of rat glypican-1 starting with ⁵¹⁰Ser. The glypican-syndecan-4 cytoplasmic domain (G1-S4c) construct was created by replacing C-terminal sequence of rat glypican-1 starting with ⁵¹⁰Ser with amino acids 247-321 of the rat syndecan-4 sequence. The created chimera thus contains both transmembrane and cytoplasmic regions of syndecan-4. Transfection of the MSCV2.2 vector alone 30 was used to generate a control ECV cell population.

Retroviral transduction

The virus for transductions was produced by calcium phosphate transient transfection (29) of 10 μ g of each construct on amphotropic Phoenix packaging cells (ATCC). Viral supernatants were collected after 36, 48 and 72 hrs, sterile 5 filtered through 0.2 μ m filter and then transferred to ECV-304 cells at 32°C in the presence of 25 μ g/ml DEAE-dextran. Typical viral titers in the supernatant were approximately 6-8 \times 10⁵ infectious particles/ml. Virus exposure was repeated 4 times for each construct; following the last exposure the cells were cultured in 10% FBS-DMEM supplemented with 400 μ g/ml active G418 (Sigma) 10 for two weeks.

Growth and migration assays

For growth assays, 100,000 cells were plated in 6 well cell culture plates and allowed to attach overnight. At that time, the cells were washed 3 times with 15 phosphate-buffered saline (PBS) and the medium was changed to DMEM supplemented with 0.25% FBS. Twenty four hours later, 25 ng/ml of bFGF (Chiron Corp.) were added to the cell culture medium. Cell counts were then obtained at 24 hr intervals starting with the time of exposure to bFGF by trypsinizing the well and counting cell suspensions on a Coulter counter (Coulter 20 Corp.).

Migration assays were carried out using modified Boyden chambers (Neuroprobe, Inc.). ECV 304 cells and derived clones were grown in 10% FBS-DMEM supplemented with 5 ng/ml DiI (DiIC₁₈; 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanide perchlorate, Molecular Probes) living cell 25 fluorescent stain overnight. Following that, the cells were trypsinized, washed with DMEM, diluted in DMEM supplemented with 0.5% FBS and seeded in wells at 60,000 cells per well. The cell containing compartments were separated from the lower wells by 25 x 80 mm polycarbonate filters with 8 μ m pores (Poretics Corp.). The lower chambers were filled with 0.5% FBS-DMEM 30 supplemented with 50 ng/ml bFGF and the entire apparatus was incubated in a tissue culture incubator at 37°C, 5% CO₂ for 4.5 hours. After that time non-migrating cells were removed by washing the upper wells with PBS, the upper

- 32 -

surfaces of the filters were scraped with a plastic blade, and the filters were fixed in 4% formaldehyde for 1 min and placed on a glass slide. The migrated cells were imaged using a digital SesSys camera attached to a Nikon fluorescent microscope. For each slide, 3 non-overlapping lower power (5x) fields were 5 selected for analysis. Following image acquisition using PMIS image processing software (Photometrics, Ltd.) the number of cells was automatically determined using Optimas 6.0 software (Bioscan, Inc.).

Matrigel growth assay

10 Growth factor depleted Matrigel (Becton Dickinson) plates were prepared by adding 0.5 ml of thawed Matrigel to a well of refrigerated 24 well tissue culture plate. The gel was allowed to solidify for one hour at 37°C and overlaid with 1 ml of 0.5% FBS-DMEM containing 30,000 cells. The cell culture was carried out at 37°C in a humidified atmosphere supplemented with 5% CO₂. The 15 analysis of cell growth was carried out by obtaining lower (10x) and high (40x) power images of the wells with a digital SesSys camera focused on the surface of the gel using an inverted Nikon fluorescent microscope. The cell-free area was the determined using Optimas 6.0 software.

20 RNA Isolation and RT PCR Analysis

For RNA analysis of syndecan-4 and PR-39 expression, cell cultures were trypsinized, pelleted, and total RNA was prepared using TRI Reagent (Sigma Biosciences). The RNA pellet was dissolved in RNase-free water and ethanol precipitated. For RT-PCR analysis, 0.2 μ g total RNA were used for reverse 25 transcription with a 15 pmol of oligo(dT)₂₀ primer, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM each dNTP in 50 mM Tris-HCl (pH 8.3) buffer. The mixture was heated to 70°C for 10 min, then cooled to 37°C while 1 μ l of Super Script II reverse transcriptase (200 U/ μ l, Life Technologies, Inc.) was added. The reaction was allowed to proceed for 1 hr at 37°C and then terminated by 30 heating for 5 min followed by chilling to 4°C. 1 μ l of the RT reaction mixture was used for PCR amplification using specific primers. The PCR reaction was carried out in the presence of 1.5 mM MgCl₂, 0.2 mM dNTP, 400 nM 3' and 5'

- 33 -

primers and 2.5 U of Taq DNA polymerase (Boehringer Mannheim, Inc.). The following specific primers were used: Glypican-1: 5': CCC CGC CAG CAA GAG CCG GAG CT; 3': GTG AGG CTC TGG GCG AGT GGG GG, 5 Syndecan-4: 5' (with Sac I restriction site): ATA GAG CTC TTG GAA CCA TGG CFC CTG TCT GCC; 3': (with Eco RI restriction site): GGA ATT CCA GGT TTT ATT ATC TTT TTA TC.

For standardization purposes a conserved region of human and mouse GAP-DH gene was chosen for amplification as a control template. The following primers were used: 5': CGT ATT GGG CGC CGT GTC ACC AGG GC; 3': 10 GGC CAT GAG CTC CAC CAC CCT GTT CG. All PCR reactions were carried out using GeneAmp PCR 2400 system (Perkins Elmer, Inc.) as follows: 94°C (1 min), 50-55°C (30 sec), 72°C (1.5 min). The additional final extension step was performed at 72°C for 7 min. A total of 30 cycles were done for each 15 reaction. Following PCR amplification, reaction products were subjected to 1% agarose gel electrophoresis and the amount of specific message was expressed as a ratio to GAP-DH message.

Determination of heparan sulfate mass in cultured cells

To determine the total mass of heparan sulfate chains, endothelial cell 20 cultures were washed twice with PBS and incubated for 24 h with 2 mCi of $\text{Na}_2^{35}\text{SO}_4$ in 2 ml of a modified basal Eagle medium supplemented with 1% Neutrodoma-SP. At the end of labeling, cells are washed with cold PBS and incubated with a lysis buffer followed by centrifugation at 15,000 x g for 10 min at 4°C. Total proteoglycans (PG) are isolated from the supernatant by DEAE 25 chromatography. Glycosaminoglycans were cleaved from the total PG pool by β -elimination and the relative content of HS and CS is determined by appropriate enzyme digests with chondroitinase ABC or *Flavobacterium* heparitinase 1 and 3. Preliminary experiments on microvascular endothelial cells demonstrated that the sum of HS and CS sulfate accounted for >98% of the total PG sulfate.

30

- 34 -

Scatchard analysis of low affinity bFGF binding sites

For determination of the number and affinity of bFGF heparan sulfate binding sites, endothelial cells were grown to near confluence in 24 well dishes in 10% FBS-DMEM. After two washes with cold PBS, 200 μ l of binding buffer (25 mM HEPES, pH 7.4, 0.1% BSA, 0.05% gelatin in M199 medium), 6 \times 10⁶ cpm (0.5 ng/ml) ¹²⁵-I-bFGF (DuPont, specific activity 2000 C/mmol), and increasing amounts (0-600 ng/ml) cold bFGF were added to each well. The cells were incubated at 4°C for 2 h with gentle agitation; at the end of that time, the cells were washed three times with 1 ml PBS containing 0.1% BSA and then incubated with 1% Triton-X 100 in 5 ml water supplemented with 0.01% BSA (Sigma) for 30 min at room temperature with vigorous shaking. Following this, 0.4 ml aliquots were counted in a 1272 CliniGamma counter (LKB). Cell counts determined by a Coulter Counter were employed to establish the number of cells per well. Background counts were subtracted from all samples. Scatchard analysis of the specifically bound material vs. the molar amount of cold competitor was carried out using Origin 5.0 software (Microcal Software, Inc., Northampton, MA). All experiments were carried in triplicate and repeated at least twice.

20

B. Empirical Data and Results

Experimental Series I

25 This series of experiments is directed to demonstrating the role of cell associated heparan sulfate chimeric core proteins in endothelial cells in-situ. The bulk of the experiments and empirical data in this series are in-vitro results.

Experiment 1:

30 The immortalized human endothelial cell line ECV304 was transfected with prepared retroviral constructs containing full length cDNAs for either syndecan-4 or glypican-1. In addition, in order to differentiate potential

- 35 -

5 biological effects secondary to increased mass of cell surface and/or extracellular heparan sulfates versus increased core protein expression, two additional chimera core protein constructs were created. In one, S4-GPI, syndecan 4 extracellular domain was linked to the glycan 1 GPI anchored; and in another, G1-S4c, the extracellular domain of glycan 1 was linked to the transmembrane and cytoplasmic domains of syndecan-4. Cells transfected with a vector only construct (ECV-VC) were used as control. Increased expression of both syndecan-4 and glycan-1 constructs was expected to result in larger numbers of heparan sulfate chains on the cell surface.

10 Subsequently, the total mass of heparan sulfate chains on the wild type as well as the 4 newly generated transfected ECV cell lines was determined. Total heparan sulfate mass was significantly increased (per μ g of total cellular protein) in ECV-S4, ECV-G1, ECV-S4-GPI and ECV-G1-S4c but not ECV-VC cells. This data is presented by Table E1.

15 In order to assess whether these changes in HS expression resulted in selective alterations of heparan binding growth factors, the low affinity binding of bFGF, a prototypical heparin binding growth factor was examined. Scatchard analysis of the wild type and newly generated transfected ECV cell lines showed that there were no significant changes in the affinity of bFGF binding (see Table E2; mean of 3 experiments). At the same time, there was a 2-fold increase in the number of bFGF binding sites in S4 and C1-S4c clones and somewhat smaller increase in ECV-G1 and ECV-S4-GPI clones (Table E2). The smaller increase in cell-associated HS mass in glycan and syndecan-4 GPI overexpressers was expected given higher shedding rates for GPI-linked glycan compared to the transmembrane syndecan. Also, the increase in the number of bFGF binding sites was of the same order as the increase in the total HS cell mass -- thus showing that there was no preferential creation of bFGF binding sites and, there was no significant change in the bFGF-HS/HS ratio (calculated as ratio of a relative increase in the number of HS-bFGF sites per cell and a relative increase in the total HS mass). Thus, for a ECV-S4 clone compared to control, there was

- 36 -

a $5.94 \times 10^6 / 2.32 \times 10^6 = 2.56$ fold increase in the number of bFGF-HS sites (Table E2) and a $0.33 / 0.14 = 2.36$ increase in the total HS mass (per μg protein, Table E1) giving the HS-bFGF/HS ratio of $2.36 / 2.56 = 0.75$.

- 37 -

Table E1: HS Mass In Various Stable Clones

<u>³⁵S HS / g protein</u>		
5	ECV-VC	0.14±0.026
	ECV-S4	0.33±0.042
	ECV-G1	0.23±0.015
	ECV-S4-GPI	0.24±0.080
	ECV-G1-S4c	0.34±0.050

10

³⁵S counts in HS expressed per g of total protein.

15

Table E2: Effect of S4, G1 and chimera constructs expression on low affinity Kd and the number of binding sites for bFGF

20		Kd	Number of sites	HS-bFGF / Total
			<u>per cell</u>	<u>HS Ratio</u>
	ECV-VC	0.60 * 10 ⁻⁹	2.32 * 10 ⁶	1.00
	ECV-S4	0.85 * 10 ⁻⁹	5.94 * 10 ⁶	0.92
	ECV-G1	0.81 * 10 ⁻⁹	3.60 * 10 ⁶	0.95
25	ECV-S4-GPI	0.69 * 10 ⁻⁹	3.80 * 10 ⁶	0.96
	ECV-G1-S4c	0.53 * 10 ⁻⁹	4.89 * 10 ⁶	0.87

Experiment 2:

To study the effect of syndecan-4 and glypican-1 expression on endothelial cell growth, the ability of wild type and newly created ECV cell lines to grow in-vitro in response to serum and bFGF was analyzed. Experimentally, all cells
5 were growth arrested for 48 hours and then stimulated with 0.25% FBS supplemented with 25 ng/ml bFGF. The data is shown by Fig. 14 in which, MSCV-ECV-vector control; G1: glypican 1 full length cDNA; S4-GPI; syndecan-4 extracellular domain linked to the GPI anchor; S4: full length syndecan-4 cDNA; G1-S4c: extracellular domain of glypican-1 linked to syndecan-4
10 transmembrane/ cytoplasmic domain.

As shown therein, the ECV-S4 and ECV-G1-S4c cells demonstrated a 4-fold increase in cell number compared to ECV wild type or vector-transfected (MSCV) cells. At the same time, growth of ECV-G1 or ECV-S4-GPI cells did not differ significantly from wild type ECV cells. Even though both ECV-G1
15 and ECV-S4-GPI clones had somewhat smaller numbers of bFGF-HS binding sites per cell, the absence of any significant change in bFGF growth response is out of proportion to the magnitude of HS-bFGF increase.

Experiment 3:

20 To test the effect of these constructs expression on the cells ability to form vascular structures, wild type and newly generated ECV clones were plated on Matrigel in 10% FBS-DMEM. Three days later, the presence of definable structures (cords and rings) was assayed by light microscopy. As in the case of in-vitro growth assays, ECV-S4 and ECV-G1-S4c cells formed more numerous
25 and denser vascular structures compared to wild type ECV, ECV-G1 or ECV-S4-GPI cells. The results are shown by Figs. 15A-15C.

As seen in Figs. 15A-15C respectively, vector transduced ECV cells (MSCV) as well as ECV transduced with full length syndecan-4 and G1-S4c construct-carrying retroviruses were plated on growth factor depleted Matrigel
30 supplemented with 25 ng/ml bFGF. Photographs of the gels were taken 72 hours later. Note the presence of increased vascular networks and cell density in S4 and G1-S4c panels compared to MSCV panel.

Experiment 4:

To further analyze the effect syndecan, glypcan, or syndecan/glypcan chimeras expression on biological behavior of endothelial cells, the migration of wild type and generated ECV cell lines migration towards serum and bFGF in 5 Boyden chamber assays was analyzed. Similar to the growth assay results, the cell lines expressing increased amounts of syndecan-4 or glypcan-syndican-4 cytoplasmic tail chimeras demonstrated a significantly higher ability to migrate compared to wild type ECV or ECV expressing glypcan-1 or extracellular domain of syndecan-4 linked to the glypcan-1 GPI anchor. This is shown by 10 Fig. 16.

Overall Conclusions:

The experiments demonstrate, therefore, that syndecan-4 expression resulted in significant increase in bFGF-stimulated growth of EC in 2-D and 3-D 15 cultures as well as in enhanced migration towards the bFGF gradient. These results cannot be attributed to the increase in HS cell mass or preferential creation of low affinity (HS) bFGF binding sites rather than increased syndecan-4 core protein expression, since overexpression of glypcan-1 while producing the same increase in HS mass did not produce increased growth and migration responses to 20 bFGF. This conclusion is further supported by observation that while glypcan- S4 cytoplasmic domain chimera closely mimicked effects of syndecan-4 overexpression, syndecan-4-GPI chimera had no effect on bFGF responses in these cells. Finally, while both syndecan-4 and glypcan1 expression increased 25 total HS cell mass there was no significant change in the number of low or high (data not shown) affinity HS bFGF binding sites. Thus, increased expression of syndecan-4 cytoplasmic domain is associated with increased responsiveness to bFGF stimulation as defined by cell growth and migration assays.

- 40 -

Experimental Series II

The second experimental series is directed to demonstrating the role of chimeric cone proteins in stimulating angiogenesis under in-vivo conditions. The 5 experiments and data presented hereinafter are representative of clinical conditions and medical pathologies in living humans and animals.

Experiment 5:

To demonstrate the role and effect of chimeric cone protein in regulation 10 of angiogenesis in-vivo, a rat myocardial infarction model [as reported in Li *et al.*, *Am. J. Physiol.* **270**: H1803-H1811 (1997)] was adapted to in-vivo studies using mice.

In this model, ligation of a proximal coronary artery leads to reproducible infarction accompanied by peri-infarction angiogenesis that can be characterized 15 in a number of ways including in-situ hybridization, immunocytochemistry and morphometric analysis. Using this model, rapid (within 1 hour) induction of syndecan-4 gene expression in peri-infarct region that was dependent on the influx of blood-derived macrophages was demonstrated. A comparison of the extent of angiogenesis in macrophage-deficient homozygous op/op mice (low post-MI 20 syndecan expression) to that in the op/op mice treated with GM-CSF (thus restoring macrophage population and syndecan-1/4 expression) revealed a 4 fold increase in neovascularization in the latter as determined by BudR intake and morphometric analysis. This result is shown by Figs. 17A-17F respectively.

Figs. 17A-17F show BudR uptake in op/op homozygous (-/-) and 25 heterozygous (+/-) mice over 3 days time. Note the intense BudR uptake by cells on the infarct periphery in (+/-) mice but not in (-/-) mice within the peri-infarct area on both day 1 and day 3 post-infarction.

Experiment 6:

30 To further link syndecan expression to enhanced angiogenic response in these settings, transgenic mice lines were generated with cardiac myocyte-specific expression of PR-39 peptide using α -MHC promoter. The PR-39 peptide has

- 41 -

been shown to increase both syndecan-1 and syndecan-4 expression in-vitro in a variety of cell types. [See for example, Gallo *et al.*, Proc. Natl. Acad. Sci. USA 91: 11035-11039 (1994) and Li *et al.*, Circ. Res. 81: 785-796 (1997)].

Analysis of syndecan gene expression in PR-39 transgenic mice
5 demonstrated marked increase in expression of syndecan-4 and glypican-1 genes. This is shown by Fig. 18. Equally important, there was no detectable expression of syndecan-1 in either wild type or transgenic mice (data not shown).

10 Immunocytochemical analysis with anti-CD31 antibody demonstrated increased vascular density in PR-39 transgenics and the morphometric analysis confirmed a 3 fold increase in the number of capillaries and small (<200 μ m diameter) diameter vessels in these mice.

15 In particular, Fig. 18 shows a Northern blot analysis of gene expression in PR-39 transgenic mice. The LV myocardium from the wild type (WT) and two PR-39 transgenic lines (A,B) mice was subjected to Northern blot analysis. Note the increased syndecan-4 and glypican-1 expression in both transgenic mice compared to WT mice.

Experiment 6:

20 To confirm the functional significance of this increase in vascularity, the total coronary resistance was assessed in an isolated heart preparation as previously described [Li *et al.*, J. Clin. Invest. 100: 18-24 (1997)]. In these settings, a 2 fold decrease in coronary perfusion pressure was observed for any given perfusion rate, thus confirming a reduced transmyocardial resistance to flow. To further evaluate vascular function in these mice, a study of bFGF-
25 induced vasodilation in microvascular preparations in-vitro demonstrated an increased bFGF sensitivity of PR-39 mice vessels. This is shown by the data of Fig. 19.

30 As presented, Fig. 19 provides an in-vitro assessment of microvascular reactivity. Microvascular preparations from PR-39 transgenic and control mice were preconstricted with endothelin and then evaluated for a vasodilatory response to an endothelium-dependent agents ADP and bFGF. Note that while

- 42 -

both PR-39 transgenics and controls are equally responsive to ADP, bFGF response is much more profound in the PR-39 mice (* p<0.05).

Overall Conclusions:

5 Myocardial-specific expression of PR-39 resulted in increased expression of syndecan-4 and glypican-1 genes that was accompanied by a functionally significant increase in coronary vascularity and enhanced bFGF responsiveness. These studies, therefore, provide rational evidence and direct support for the in-vivo efficacy of chimeric cone protein expression in angiogenic stimulation.

10

The present invention is not to be limited in scope nor restricted in form except by the claims appended hereto.

What we claim is:

1. A prepared DNA segment for placement in a suitable expression vector and transfection of endothelial cells in-situ such that overexpression of
5 extracellular matrix heparan sulfate proteoglycan entities subsequently occurs in-situ, said prepared DNA segment comprising:
 - at least one first DNA sequence coding for the extracellular domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said extracellular domain first DNA sequence specifying the extracellular N-
10 terminal portion of an expressed proteoglycan entity which is then located at and extends from the endothelial cell surface and is capable of binding heparan sulfates to form an extracellular matrix in-situ;
 - at least one second DNA sequence coding for the transmembrane domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell
15 in-situ, said transmembrane domain second DNA sequence specifying the medial portion of an expressed proteoglycan entity which is then located at and extends through the endothelial cell membrane and is joined with said extracellular N-terminal portion of said expressed proteoglycan entity; and
 - at least one third DNA sequence coding for the cytoplasmic domain of the
20 syndecan-4 molecule in said discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said syndecan-4 cytoplasmic domain third DNA sequence specifying the cytoplasmic portion of an expressed proteoglycan entity which is then present within the cytoplasm of a transfected endothelial cell and is joined to said transmembrane portion and said extracellular N-terminal
25 portion of said expressed proteoglycan entity.
2. A constructed expression vector for transfection of endothelial cells in-situ such that overexpression of extracellular matrix heparan sulfate proteoglycan entities subsequently occurs in-situ, said constructed expression vector
30 comprising:

- 44 -

a prepared DNA segment comprised of

(i) at least one first DNA sequence coding for the extracellular domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said extracellular domain first DNA sequence specifying the extracellular N-terminal portion of an expressed proteoglycan entity which is then located at and extends from the endothelial cell surface and is capable of binding heparan sulfates to form an extracellular matrix in-situ,

(ii) at least one second DNA sequence coding for the transmembrane domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said transmembrane domain second DNA sequence specifying the medial portion of an expressed proteoglycan entity which is then located at and extends through the endothelial cell membrane and is joined with said extracellular N-terminal portion of said expressed proteoglycan entity, and

(iii) at least one third DNA sequence coding for the cytoplasmic domain of the syndecan-4 molecule in said discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said syndecan-4 cytoplasmic domain third DNA sequence specifying the cytoplasmic portion of an expressed proteoglycan entity which is then present within the cytoplasm of a transfected endothelial cell and is joined to said transmembrane portion and said extracellular N-terminal portion of said expressed proteoglycan entity; and

an expression vector carrying said prepared DNA segment and suitable for transfection of endothelial cells in-situ.

3. An in-situ transfected endothelial cell which overexpresses extracellular matrix heparan sulfate proteoglycans and positions on the proteoglycan entities at the cell surface, said in-situ transfected endothelial cell comprising:

a viable endothelial cell previously transfected in-situ with a constructed expression vector such that said transfected endothelial cell overexpresses discrete extracellular matrix heparan sulfate proteoglycan entities coded for by said vector, said overexpressed proteoglycan entities being comprised of

- 45 -

(i) an extracellular N-terminal portion which is located at and extends from the transfected endothelial cell surface and which binds heparan sulfates to form an extracellular matrix in-situ, said extracellular N-terminal portion being the expressed product of at least one first DNA sequence in the 5 prepared expression vector coding for the extracellular domain of said proteoglycan entity expressed by the transfected endothelial cell in-situ,

(ii) a transmembrane medial portion which is located at and extends through the endothelial cell membrane and is joined with said extracellular N-terminal portion of said proteoglycan entity, said transmembrane 10 medial portion being the expressed product of at least one second DNA sequence in the prepared expression vector coding for the transmembrane domain of said proteoglycan entity expressed by the transfected endothelial cell in-situ, and

(iii) a syndecan-4 cytoplasmic portion present within the cytoplasm of the transfected endothelial cell which is joined to said 15 transmembrane portion and said extracellular N-terminal portion of said proteoglycan entity, said syndecan-4 cytoplasmic portion being the expressed product of at least one third DNA sequence in the prepared expression vector coding for the cytoplasmic domain of the syndecan-4 molecule of said proteoglycan entity expressed by the transfected endothelial cell in-situ.

20

4. The prepared DNA segment as recited by claim 1 wherein said first DNA sequence coding for the extracellular domain of a discrete proteoglycan entity is selected from the group consisting of syndecan DNA sequences, glypican DNA sequences and perlecan DNA sequences.

25

5. The prepared DNA segment as recited by claim 1 wherein said second DNA sequence coding for the transmembrane domain of a discrete proteoglycan entity is selected from the group consisting of syndecan DNA sequences, glypican DNA sequences and perlecan DNA sequences.

30

6. The constructed expression vector as recited by claim 2 wherein said expression vector suitable for transfection of endothelial cells in-situ is a plasmid.

- 46 -

7. The constructed expression vector as recited by claim 2 wherein said expression vector suitable for transfection of endothelial cells in-situ is a virus.

8. The in-situ transfected endothelial cell as recited by claim 3 wherein said cell is selected from the group consisting of vascular endothelial cells and dermal endothelial cells.

9. The in-situ transfected endothelial cell as recited by claim 3 wherein said cell exists under in-vivo conditions.

10. The in-situ transfected endothelial cell as recited by claim 3 wherein said cell exists under in-vitro conditions.

11. The in-situ transfected endothelial cell as recited by claim 3 wherein said transfected endothelial cell exists in a tissue comprising at least one kind of muscle cell selected from the group consisting of myocardial muscle cells, smooth muscle cells and striated muscle cells.

12. A method for making a prepared DNA segment intended for placement in a suitable expression vector and transfection of endothelial cells in-situ such that overexpression of extracellular matrix heparan sulfate proteoglycan entities subsequently occurs in-situ, said method comprising the steps of:

obtaining at least one first DNA sequence coding for the extracellular domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said extracellular domain first DNA sequence specifying the extracellular N-terminal portion of an expressed proteoglycan entity which is then located at and extends from the transfected endothelial cell surface and is capable of binding heparan sulfates to form an extracellular matrix in-situ;

acquiring at least one second DNA sequence coding for the transmembrane domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said transmembrane domain second DNA sequence specifying the medial portion of an expressed proteoglycan entity which

- 47 -

is then located at and extends through the transfected endothelial cell membrane and is joined with said extracellular N-terminal portion of said expressed proteoglycan entity;

procuring at least one third DNA sequence coding for the cytoplasmic domain of the syndecan-4 molecule in said discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said syndecan-4 cytoplasmic domain third DNA sequence specifying the cytoplasmic portion of an expressed proteoglycan entity which is then present within the cytoplasm of a transfected endothelial cell and is joined to said transmembrane portion and said extracellular N-terminal portion of said expressed proteoglycan entity; and
5 joining together said extracellular domain first DNA sequence, said transmembrane domain second DNA sequence, and said syndecan-4 cytoplasmic domain third DNA sequence as a discrete prepared DNA segment.

10 13. A method for making a constructed expression vector intended for transfection of endothelial cells in-situ such that overexpression of extracellular matrix heparan sulfate proteoglycans subsequently occurs in-situ, said method comprising the step of:

15 obtaining a prepared DNA segment comprised of
20 (i) at least one first DNA sequence coding for the extracellular domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said extracellular domain first DNA sequence specifying the extracellular N-terminal portion of an expressed proteoglycan entity which is then located at and extends from the transfected endothelial cell surface and is capable of binding heparan sulfates to form an extracellular matrix in-situ,
25 (ii) at least one second DNA sequence coding for the transmembrane domain of a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said transmembrane domain second DNA sequence specifying the medial portion of an expressed proteoglycan entity which is then located at and extends through the transfected endothelial cell membrane and is joined with said extracellular N-terminal portion of said expressed proteoglycan entity, and
30

- 48 -

(iii) at least one third DNA sequence coding for the cytoplasmic domain of the syndecan-4 molecule in a discrete proteoglycan entity that is expressed by a transfected endothelial cell in-situ, said syndecan-4 cytoplasmic domain third DNA sequence specifying the cytoplasmic portion of an expressed proteoglycan entity which is then present within the cytoplasm of a transfected endothelial cell and is joined to said transmembrane portion and said extracellular N-terminal portion of said expressed proteoglycan entity; and

5 positioning said prepared DNA segment in an expression vector suitable for transfection of endothelial cells in-situ.

10

14. A method for stimulating angiogenesis in-situ within a living tissue comprising vascular endothelial cells, said method comprising the steps of:

transfected vascular endothelial cells within a living tissue with a constructed expression vector such that the resulting transfected endothelial

15 vascular cells overexpress discrete extracellular matrix heparan sulfate proteoglycan entities coded for by said constructed expression vector, said overexpressed proteoglycan entities being comprised of

(i) an extracellular N-terminal portion which is located at and extends from the transfected vascular endothelial cell surface and binds heparan

20 sulfates to form an extracellular matrix in-situ, said extracellular N-terminal portion being the expressed product of at least one first DNA sequence in the constructed expression vector coding for the extracellular domain of said proteoglycan entity expressed by a transfected endothelial cell in-situ,

(ii) a transmembrane medial portion which is located at and extends through a transfected vascular endothelial cell membrane and is joined with said extracellular N-terminal portion of expressed proteoglycan entity, said transmembrane medial portion being the expressed product of at least one second DNA sequence in the constructed expression vector coding for the transmembrane domain of said proteoglycan entity expressed by a transfected endothelial cell in-

25 situ, and

30

- 49 -

(iii) a syndecan-4 cytoplasmic portion present within the cytoplasm of a transfected endothelial cell which is joined to said transmembrane portion and said extracellular N-terminal portion of said proteoglycan entity, said syndecan-4 cytoplasmic portion being the expressed product of at least one third
5 DNA sequence in the constructed expression vector coding for the cytoplasmic domain of the syndecan-4 molecule of said proteoglycan entity expressed by a transfected endothelial cell in-situ; and
allowing said transfected vascular endothelial cells bearing said
overexpressed extracellular matrix proteoglycan entities to stimulate angiogenesis
10 in-situ.

15. The method for stimulating angiogenesis in-situ as recited by claim 14 wherein said living tissue comprises at least one other type of cell selected from the group consisting of muscle cells, fibrocytes and fibroblasts, epithelial cells, osteocysts and osteoblasts, erythrocytes and leukocytes, and neurons.

16. The method for stimulating angiogenesis in-situ as recited by claim 14 wherein said living tissue comprises at least one tissue selected from the group consisting of myocardium, lung, brain, kidney, spleen, liver, and gastro-intestinal
20 tissues.

17. The method for stimulating angiogenesis in-situ as recited by claim 14 wherein said living tissue comprising vascular endothelial cells is transfected using means selected from the group consisting of catheter-based administration,
25 injection-based administration, infusion-based administration, localized intravascular deliveries, liposome-based deliveries, and administrations using target-directed peptides.

18. The method for stimulating angiogenesis in-situ as recited by claim 14
30 wherein said method is practiced under in-vivo conditions.

- 50 -

19. The method for stimulating angiogenesis in-situ as recited by claim 14
wherein said method is practiced under in-vitro conditions.

Fig. 1

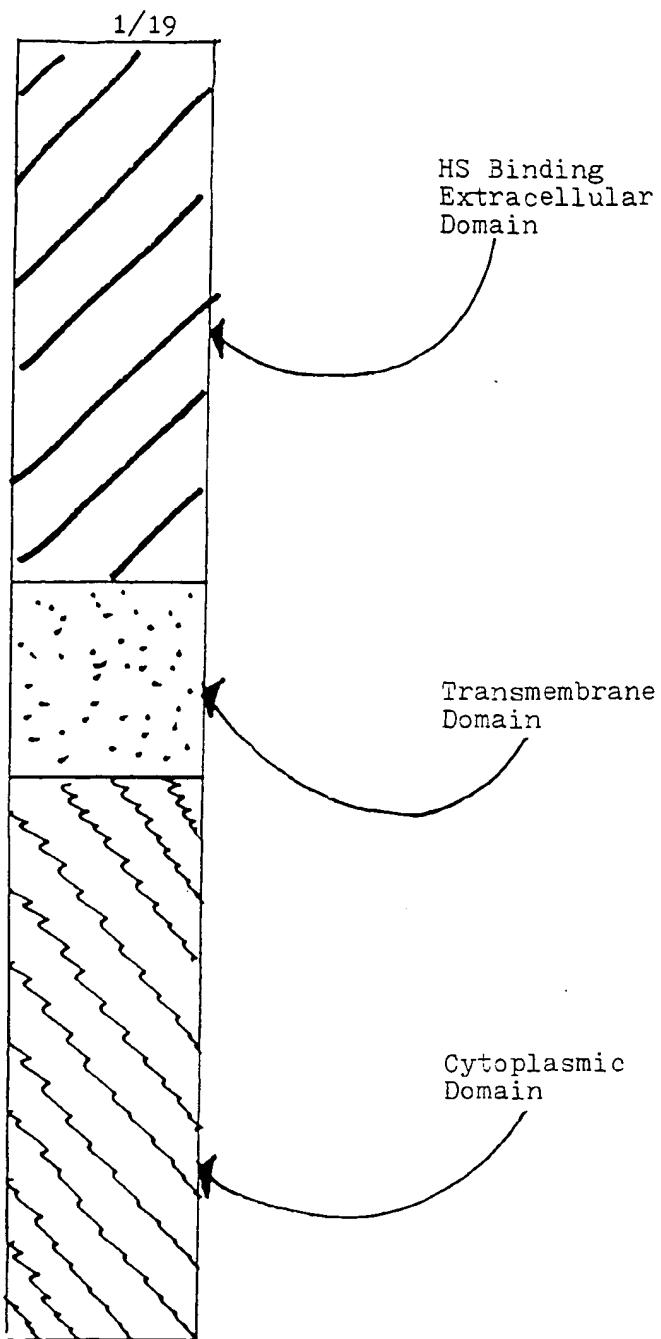


Fig. 2: Syndecan-1 (rat) extracellular domain:

```

atgagac gtgcggcgct
ctggcttgg ctctgcgcgc tggcgctgcg cctgcagcct gccctccgc aaattgtcac
cgaaatgtg cctcttgaag accaagatgg ctctggggac gactcagaca acttctctgg
ctcaggcaca ggtgcttgc cagatatgac tttgtcacgg cagacacctt ccacttggaa
ggatgtgtgg ctctgtacag ctacacccac agtccagaa cccaccagca gggataccga
ggccaccctc acctctatcc tgccggctgg agagaagcct gaggagggag agcccgtggc
ccacgtgaa gcagagcctg acttcaactgc tggggacaag gagaaggagg ccaccaccag
gccttagggag accacacagc tcccagtac ccaacaggcc tcaacagcag ccagagccac
cacggccag gcatctgtca cgtctcatcc ccacggggat gtgcaacctg gcctccacga
gaccttgct cccacagcac ccggccaacc tgaccatcaag cctccaaatgtg tggaggatgg
aggcacttct gtcatcaaag aggttgtgga ggatgaaact accaatcagc ttctgcagg
agagggtct ggagaacaag acttcacctt taaaacatct gggagaaca cagctgtggc
tggcgtcgag cctgaccttc ggaatcagtc cccagtggat gaaggagcca caggtgcttc
tcagggcctt ttggacagga aggaa

```

ACCESSION M81785
 LOCUS RATSYNDECA 2396 bp mRNA ROD 16-JUL-1992
 DEFINITION Rattus norvegicus syndecan mRNA, complete cds.
 ACCESSION M81785
 NID 9207140
 KEYWORDS syndecan.
 SOURCE Rattus norvegicus Epididymal fat pad cDNA to mRNA.
 ORGANISM Rattus norvegicus
 Eukaryota; mitochondrial eukaryotes; Metazoa; Chordata;
 Vertebrata; Eutheria; Rodentia; Sciurognathi; Myomorpha; Muridae;
 Murinae; Rattus.
 REFERENCE 1 (bases 1 to 2396)
 AUTHORS Kojima,T., Shworak,N.W. and Rosenberg,R.D.
 TITLE Molecular cloning and expression of two distinct cDNA-encoding
 heparan sulfate proteoglycan core proteins from a rat endothelial
 cell line
 JOURNAL J. Biol. Chem. 267, 4870-4877 (1992)

3/19

Fig. 3: Syndecan-2 (human) extracellular domain

ggcaggagggg agggagccag agaaaaagaa gaggaggaga aggaggagga cccggggagg
 gagggcggc gccccggag gaggggcgca gcccggcgc cagtggccccc gttggacgc
 gctgtctcc agataccccc ggagctccag cccgcggat cggcgctcc cggcgctctg
 cccctaaact tctgcctgt ctcctttca agccagcga ttatccctt aaaaccagaa
 actgaacctc ggcacggaa aggagtccgc ggaggagca aaccacagca gagcaagaag
 agcttcagag agcagcctc cccggagcacc aactccgtgt cgggagtgca gaaaccaaca
 agtgagaggg cggcggttc cccggggcgc gctgccccgg gcccggagcag gcccggagg
 aggaagcgg cccccccgg ccccgagccc gagtccccga gcctgagccg caatcgctgc
 ggtactctgc tccggatcg tggcgccgg ctcggcggc gctggggcagg aggttcgtt
 ttggccctggg tgcaagcgc ggctgggagc agccgggtcc tgggaaatat gggcgccgc
 tggatctgc tcaccttggg cttgggtggcc tgcgtgtcgg cggagtccgag agcagagctg
 acatctgata aagacatgt aacttgacaac agctccattg aagaagcttc aggagtgtat
 cctattgtg acgatgacta cgcttctgcg tctggctcg gagctgtatga ggatgttagag
 agtccagagc tgacaacaac tcgaccactt ccaaagatac tggacttag tgctgctcca
 aaagtggaaa ccacgacgt gaatatacag aacaagatac ctgctcagac aaagtccac
 gaagaaaactg ataaagagaa agttcaccc tctgacttag aaaggaaaat ggaccagcc
 gaagaggata caaatgtgt tactgagaaa cactcagaca gtctgtttaa acggacagaa

protein sequence:

GRREGARGKEEEKEEOPGREARRRRRGAAAEPVAPLGRAALQ
 IPPELQPRGSRAAALPLNFCRSLSQRIYSLKPEPEPRHGKGVRRGAKPQQSKKS
 REQPSRSTNSVSGVQKPTSERAPRSRGAAAGGGSSRRRRKRAAPSPPEPESPLSRNRC
 GTLLRIRVGLAERWAGGFVLPMLOAAAGSSRSLGNNRRAWILLTGLVACVSAERSA
 ELTSOKMVLNDSIEEASGVYPIDDDYASASGSGAODEDVESEPLTTTRPLPKILLT
 SAAPKETTLNQNKIPQTKSPEETDKEKVHLSOSERKMDPAEEDTNVYTEKHSDS
 LFKRTE

reference:

ACCESSION J04621
 LOCUS HUMHSPGC 3414 bp mRNA PRI 08-NOV-1994
 DEFINITION Human heparan sulfate proteoglycan (HSPG) core protein, 3' end
 ACCESSION J04621
 NID 9184428
 KEYWORDS core protein; heparan sulfate proteoglycan.
 SOURCE Human fetal lung fibroblast, cDNA to mRNA, clone 48K5.
 ORGANISM Homo sapiens
 Eukaryota; mitochondrial eukaryotes; Metazoa; Chordata;
 Vertebrata; Eutheria; Primates; Catarrhini; Hominidae; Homo.
 REFERENCE 1 (bases 1 to 3414)
 AUTHORS Marynen, P., Zhang, J., Cassiman, J.J., Van den Berghe, H. and David, G.
 TITLE Partial primary structure of the 48- and 90-kilodalton core
 proteins of cell surface-associated heparan sulfate proteoglycans
 of lung fibroblasts. Prediction of an integral membrane domain and
 evidence for multiple distinct core proteins at the cell surface of
 human lung fibroblasts
 JOURNAL J. Biol. Chem. 264 (12), 7017-7024 (1989)

4/19

Fig. 4: Synd-3 (N-Syndecan) extracellular domain

gccccccgcgcgtgctgagccgtccttgcggcacgssatgcccggagctgcggcgcc
 tcgcgggtgcgtgcgtgcgtcagccccgcgcagcgctggctcagccgtggcaatg
 agaactacgagaggccgggtggacctggaggctctggggatgtatcccttggggacg
 atgaactggatgacatctactcggttcggcgtcaggctatggcaggagtcagggt
 tggagacagcggtcagcctcaccacggacacgtccgtccactgcccaccacgggtggccg
 tgctgcgtgcacccctgggtgcagccatggcaacaccctttagctgttccccacagagg
 acacgtccccctgagcaaacaaccagcgtctttagtatacccaagataacagaacaccag
 tgatccccagctggaaaacaaccacccggccagtgaccactgccagtgactccccagtagcca
 cctccaccaccaccacccggctgtaccaccaccaaccaccaccatcagcacca
 ctgtggccacccccaagccaccactaccacccagagggttcctgcccccttgcaccaagg
 cagccaccacccgggaccacccctggagacgcccaccacccatccatccctgaaaccagtg
 tcctgacagaggtgaccacatcacggcttgcacccctccagcacagccaagccgaggtccc
 tgccaaaaccaaggacttccaggactgcagaacccacggaaaaaaagcactgccttcgcctt
 ccagccccaccacccgtgcacccacagaagccccccaggtggagcggggagttgacga
 cagtcctcgacagtgacccatggaaagtcccaaccagtagtggccccagcggggacttcgaga
 tccaggaggaggaggagacaactcgctctgagctggcaatgagggtggcagtggta
 caccaccagcagcaccggggctggcaagaatgcagagccgggctcatcgacaacacaa
 tagagtcggcagctggctgctcagctccccagaaaaacatcctggagaggaaggaa

Reference

ACCESSION M84910
 LOCUS CHKSNDPRO 1372 bp mRNA VRT 29-NOV-1995
 DEFINITION Chicken syndecan-3 proteoglycan mRNA, complete cds.
 ACCESSION M84910
 HID g1017461
 KEYWORDS syndecan-3 proteoglycan.
 SOURCE Gallus gallus 4-6 day and 10 day forelimb and whole embryo cDNA to mRNA.
 ORGANISM Gallus gallus
 Eukaryota; mitochondrial eukaryotes; Metazoa; Chordata;
 Vertebrata; Archosauria; Aves; Neognathae; Galliformes;
 Phasianidae; Phasianinae; Gallus.
 REFERENCE 1 (bases 1 to 1372)
 AUTHORS Gould,S.E., Upholt,W.B. and Kosher,R.A.
 TITLE Syndecan 3: a member of the syndecan family of membrane-intercalated proteoglycans that is expressed in high amounts at the onset of chicken limb cartilage differentiation
 JOURNAL Proc. Natl. Acad. Sci. U.S.A. 89 (8), 3271-3275 (1992)
 MEDLINE 92228766
 REFERENCE 2 (bases 1 to 1372)
 AUTHORS Gould,S.E., Upholt,W.B. and Kosher,R.A.
 TITLE Characterization of chicken syndecan-3 as a heparan sulfate proteoglycan and its expression during embryogenesis
 JOURNAL Dev. Biol. 168 (2), 438-451 (1995)

5/19

Fig. 5: Syndecan-4 extra-cellular domain from rat:

```
atggc gcctgtctgc ctgtttgcgc cgctgctgct gttgtccctc  
ggagggttcc ccgtcgcccc agggagtcg attcgagaga ctgaggtcat agaccccccag  
gacctcctgg aaggcagata cttctctgg a gcccctccgg acgatgaaga cgctggggc  
cttgagcagg actctgactt tgagctgtcg gttccggag atcttagatga cacggaggag  
cccagaccc tccctgaggt gatttcaccc ttgggtccac tagataacca catccccag  
aatgcccagc ctggcatccg tgtccctca gagcccaagg aactggaaga gaatgaggc  
atccccaaa gggtccctc cgacgtgggg gatgacgatg tgtccaacaa agtgtccatg  
tccagactt cccagggcag caacatttt gaaagaactg ag
```

Reference:

ACCESSION M81786
LOCUS RATRYUDOCA 2452 bp mRNA ROD 16-JUL-1992
DEFINITION Rattus norvegicus ryudocan mRNA, complete cds.
ACCESSION M81786
NID g206822
KEYWORDS ryudocan.
SOURCE Rattus norvegicus Epididymal fat pad cDNA to mRNA
ORGANISM Rattus norvegicus
Eukaryota; mitochondrial eukaryotes; Metazoa; Chordata;
Vertebrata; Eutheria; Rodentia; Sciurognathi; Myomorpha; Muridae;
Murinae; Rattus.
REFERENCE 1 (bases 1 to 2452)
AUTHORS Kojima,T., Shworak,N.W. and Rosenberg,R.D.
TITLE Molecular cloning and expression of two distinct cDNA-encoding
heparan sulfate proteoglycan core proteins from a rat endothelial
cell line
JOURNAL J. Biol. Chem. 267, 4870-4877 (1992)

6/19

Fig. 6: Glypican-1 (rat) extracellular Domain:

```

atggag
ctccggggccc gaggctggtg gctgctgtgc gcggccgccc cgctagtcgc ctgcgcccgc
ggggacccccc ccagcaagag cccggagctgc agcgaagttcc gcccagatcta cggggctaa
ggcttttagcc tgagcgacgt gcccaggca gagatotcgg gagagcacct ggggatctgc
ccccagggtc acacactgctg caccagttag atggaggaga acctggccaa ccacagccgg
atggagctgg agaccgcact ccacgacagc agccgtgccc tgcaggctac actggccacc
cagctgcatg gcatcgatga ccacttccag cgcctgtga atgactcgga gctacactg
caggatgctt ttcccggggc ctttggggac ctgtacacgc agaacaactcg ggccttccgg
gacctgtatg ctgagctgcg tctctactac cgaggggcca acctacacct tgaggagaca
ctggccgagt tctgggcacg gctgctggag cgtctcttca agcagctgca cccccagctt
ctgctgccc atgactatct gqactgcctg ggcaaggcagg cagaggcact gggccggtt
ggggatgccc ctcgagaact ggcctgagg gccacccgtg cttttgtggc ggcacgatcc
tttgtgcagg gcctgggtgt ggccagtgcg qtagtccgaa aggtggccca ggttctctcg
gccccagaat gttctcgggc tgcataaag ttggctact gtgcccattt cccgggagtc
cctggtgccc ggcctgtcc cgactattgc cgaatgtgc tcaaaggctg cttgccaac
caggccgacc tggatgcccgt gttggaggaac ctccctggact ccatggtgc catcaactgac
aagttctggg gcccgtcggg tgcggagaat gtcattggca gtgtgcataat gtggctggcg
gaggccatca acgccttcca ggacaacaag gacacactca cagctaaggat catccaggc
tgccggaaacc ccaaggctaa tccccatggc tctggcctg aggagaagcg tgcggcgtgc
aaactggcac tgcaggagaa gtcctccaca ggtactctgg aaaagctgtt ctctgaggcc
aaggcccagc tccgagacat tcaggactac tggatcagcc tcccaggagc actgtgttagt
gagaagatgg ccatgagtcc tgccagcgt gaccgctgtt ggaatggat ttccaaggc
cggtacctac ctgaggtgat ggggtatggg ctggccaacc agatcaacaa ccctgaagt
gaggtggaca tccccaagcc ggatatgacc atccggcagc agatcatgca gctcaagatc
atgaccaacc gtttacgtgg cgccctacggg ggcaatgtat tggacttcca ggatggcagg
gatgacggca gtggctccgg cagcggtggc ggatggccag atgacgcctg tggccggagg
gtcagcaaga agactccag ctcccgacc cccttgaccc atgcccctccc cggcttgc
gaacaggagg gacagaagac ctgc

```

Protein sequence:

```

MELRARGWHLCAAAALVACARGDPASKRSRSCSEVRQIYGAKE
SLSDPQAEISGEHLRIPQGYTCCTSEMEENLANSRMELETALHOSSRALQATLAT
JLHGIDDHFQRLLNDSERTLQDAFPGAFGDLYTQNTRAFRDLYAELRLYRGANLHLE
ETLAEFWARLLERLFIKQLHPQLLPPDYLDCLGKQAEALRPFGDAPRELRLRATRAFV
AARSFVQGLGVASDVVRKVAQVPLAPECNSRAVMKLVYCAHCRGVPGARCPDYCERNVL
KGCLANQADLDAEWRNLLDSMVLITDKFWGPGSAENIVGSVHMWLAEAINALQDNKDT
LTAKVIQGGCGNPVKNPNGSGPEEKRRRGKLALQEKSSTGTLEKLVSEAKAQLRDIQDY
WISLPGTLCSEKMMAMSPASDRCWNGISKGRYLPVMDGLANQINNPVEVEVDITKPD
MTIRQCIQMLKIMTNRGAYGGNDVDFQDASDDGSGSGSGGGCPDAGCRRVSKSS
SSATPLTHALPGLSEQEQKQTKSA

```

Reference:

```

ACCESSION L34067
NID 9506416
KEYWORDS glypican.
SOURCE Rattus norvegicus (strain New England Deconess Hospital) cDNA to
mRNA.
ORGANISM Rattus norvegicus
Eukaryota; mitochondrial eukaryotes; Metazoa; Chordata;
Vertebrata; Eutheria; Rodentia; Sciurognathia; Myomorpha; Muridae;
Murinae; Rattus.
REFERENCE 1 (bases 1 to 1737)
AUTHORS Litwack, E.D., Stipp, C.S., Kumbasar, A. and Lander, A.O
TITLE Neuronal expression of glypican, a cell-surface
glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan,
in the adult rat nervous system
JOURNAL J. Neurosci 14, 3713-3724 (1994)

```

7/19

Fig. 7 : Syndecan-1 (rat) transmembrane domain:

gtgct gggaggtgto attgctggag gcctgytggt
cctcatcttt gctgtgtgcc tggtggtttt catgctatac

Reference:
ACCESSION M81785
LOCUS RATSYNDCA 2396 bp mRNA ROD 16-JUL-1992
DEFINITION Rattus norvegicus syndecan mRNA, complete cds.
ACCESSION M81785
NID g207140
KEYWORDS syndecan.
SOURCE Rattus norvegicus Epididymal fat pad cDNA to mRNA.
ORGANISM Rattus norvegicus
Eukaryota; mitochondrial eukaryotes; Metazoa; Chordata;
Vertebrata; Eutheria; Rodentia; Sciurognathi; Myomorpha; Muridae;
Murinae; Rattus.
REFERENCE 1 (bases 1 to 2396)
AUTHORS Kojima,T., Shworak,N.W. and Rosenberg,R.D.
TITLE Molecular cloning and expression of two distinct cDNA-encoding
heparan sulfate proteoglycan core proteins from a rat endothelial
cell line
JOURNAL J. Biol. Chem. 267, 4870-4877 (1992)

8/19

Fig. 8 : Syndecan-2 (human) transmembrane domain

gtccttagcag ctgtcattgc tggggagtt attggcttc tcttgtcaat ttttcttata
ctgctgttgg tg

protein sequence:
VLAAVIAGGVIGFLFAIFLILLLV

reference:

ACCESSION J04621
LOCUS HUMHSPGC 3414 bp mRNA PRI 08-NOV-1994
DEFINITION Human heparan sulfate proteoglycan (HSPG) core protein, 3' end.
ACCESSION J04621
NID 9184428
KEYWORDS core protein; heparan sulfate proteoglycan.
SOURCE Human fetal lung fibroblast, cDNA to mRNA, clone 48K5.
ORGANISM Homo sapiens
Eukaryota; mitochondrial eukaryotes; Metazoa; Chordata;
Vertebrata; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE 1 (bases 1 to 3414)
AUTHORS Matyinen, P., Zhang, J., Cassiman, J.J., Van den Berghe, H. and David, G
TITLE Partial primary structure of the 48- and 90-kilodalton core
proteins of cell surface-associated heparan sulfate proteoglycans
of lung fibroblasts. Prediction of an integral membrane domain and
evidence for multiple distinct core proteins at the cell surface of
human lung fibroblasts
JOURNAL J Biol Chem. 264 (12), 7017-7024 (1989)

9/19

Fig. 9 : Synd-3 (N-Syndecan) chicken Transmembrane domain

gtgtttagatgtgtgatttcggcggtgtggggagccctttgtgccttcctgtca
tgctgctcatctac

Reference:
ACCESSION M84910
LOCUS CHKSNDPRO 1372 bp mRNA VRT 29-NOV-1995
DEFINITION Chicken syndecan-3 proteoglycan mRNA, complete cds.
ACCESSION M84910
NID g1017461
KEYWORDS syndecan-3 proteoglycan.
SOURCE Gallus gallus 4-6 day and 10 day forelimb and whole embryo cDNA to mRNA.
ORGANISM Gallus gallus
Eukaryota; mitochondrial eukaryotes; Metazoa; Chordata;
Vertebrata; Archosauria; Aves; Neognathae; Galliformes;
Phasianidae; Phasianinae; Gallus.
REFERENCE 1 (bases 1 to 1372)
AUTHORS Gould,S.E., Upholt,W.B. and Kosher,R.A.
TITLE Syndecan 3: a member of the syndecan family of membrane-intercalated proteoglycans that is expressed in high amounts at the onset of chicken limb cartilage differentiation
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 89 (8), 3271-3275 (1992)
MEDLINE 92228766
REFERENCE 2 (bases 1 to 1372)
AUTHORS Gould,S.E., Upholt,W.B. and Kosher,R.A.
TITLE Characterization of chicken syndecan-3 as a heparan sulfate proteoglycan and its expression during embryogenesis
JOURNAL Dev. Biol. 168 (2), 438-451 (1995)

10/19

Figure 10 : Syndecan-4 transmembrane domain from rat:

gtcttggc agctctgatt gtgggcggcg tagtgggcat cctcttcgccc gtttcctga
tcctgctgct ggtgtac

Reference:
ACCESSION M81786
LOCUS RATRYUDOC A 2452 bp mRNA ROD 16-JUL-1992
DEFINITION Rattus norvegicus ryudocan mRNA, complete ccs
ACCESSION M81786
NID Q206822
KEYWORDS ryudocan
SOURCE Rattus norvegicus Epididymal fat pad cDNA to mRNA.
ORGANISM Rattus norvegicus
Eukaryota; mitochondrial eukaryotes; Metazoa; Chordata;
Vertebrata; Eutheria; Rodentia; Sciurognathi; Myomorpha; Muridae;
Murinae; Rattus.
REFERENCE 1 (bases 1 to 2452)
AUTHORS Kojima, T., Shworak, N.W. and Rosenberg, R.D.
TITLE Molecular cloning and expression of two distinct cDNA-encoding
heparan sulfate proteoglycan core proteins from a rat endothelial
cell line
JOURNAL J Biol Chem. 267, 4870-4877 (1992)

11/19

Fig. 11 : Glypican-1 (rat) GPI-Transmembrane Domain:

gccgcc actcgcccaag agcctcacta cttctttctg
ctcttcctgt tcacacctggc ctttgctgca gccaggccca ggtggcggta actgccc

protein sequence:
ATRPEPHYFFLLFLFTLVLAARPRWR

Reference:
ACCESSION L34067
NID 9506416
KEYWORDS glypican.
SOURCE *Rattus norvegicus* (strain New England Deconess Hospital) cDNA to mRNA
ORGANISM *Rattus norvegicus*
Eukaryota; mitochondrial eukaryotes; Metazoa; Chordata;
Vertebrata; Eutheria; Rodentia; Sciurognathi; Myomorpha; Muridae;
Murinae; *Rattus*.
REFERENCE 1 (bases 1 to 1737)
AUTHORS Litwack, E.D., Stipp, C.S., Kumbasar, A. and Lander, A.D.
TITLE Neuronal expression of glypican, a cell-surface
glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan,
in the adult rat nervous system
JOURNAL J Neurosci. 14, 3713-3724 (1994)

Figure 12 : Perlecan (human) transmembrane domain #M85289

tcgcgacactgtctatcccg
 ccatcaggactgtgacgcccgttctaccccttgctggccaccaggccatgtgcggactg
 cc

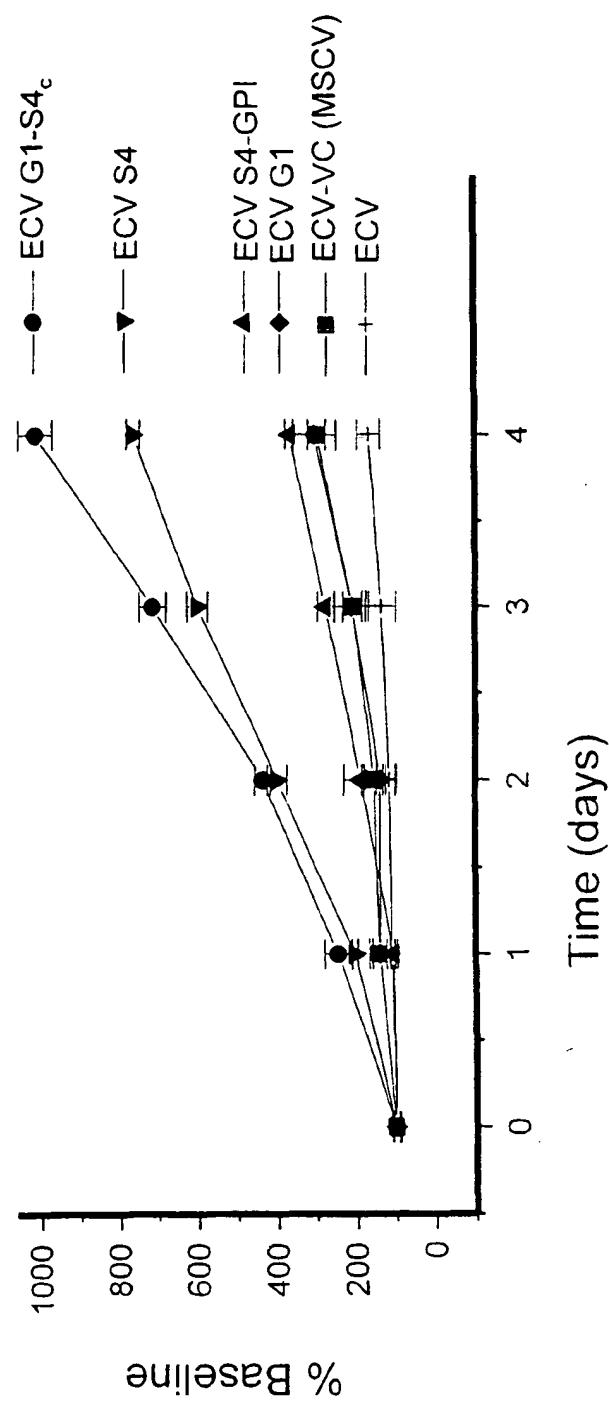
Reference: HUMHSPG2B 14327 bp mRNA PRI 08-NOV-1994
 LOCUS Human heparan sulfate proteoglycan (HSPG2) mRNA, complete cds.
 DEFINITION M85289
 ACCESSION M85289
 NID 9184426
 KEYWORDS HSPG2 gene; heparan sulfate proteoglycan.
 SOURCE Homo sapiens skin; colon cDNA to mRNA.
 ORGANISM Homo sapiens
 Eukaryote; mitochondrial eukaryotes; Metazoa; Chordata;
 VERTEBRATA; Eutheria; Primates; Catarrhini; Hominidae; Homo.
 REFERENCE 1 (bases 1 to 1427)
 AUTHORS Dodge, G. R., Kovalszky, I., Chu, M. I., Hasseit, J. R., McBride, O. M.,
 Yi, H. F. and Iozzo, R. V.
 TITLE Heparan sulfate proteoglycan of human colon: partial molecular
 cloning, cellular expression, and mapping of the gene (HSPG2) to
 the short arm of human chromosome 1
 JOURNAL Genomics 10 (3), 673-680 (1991)
 MEDLINE 91365376
 REFERENCE 2 (bases 1 to 1427)
 AUTHORS Murdoch, A. D., Dodge, G. R., Cohen, J., Tuan, R. S. and Iozzo, R. V.
 TITLE Primary structure of the human heparan sulfate proteoglycan from
 basement membrane (HSPG2/perlecan). A chimeric molecule with
 multiple domains homologous to the low density lipoprotein
 receptor, laminin, neural cell adhesion molecules, and epidermal
 growth factor
 J Biol Chem. 267 (12), 8544-8557 (1992)

Figure 13 : Syndecan-4 cytoplasmic domain from rat: M81786

Cgc atgaagaaga aggatgaagg cagttacgac ttggccaaga aaccctatca caaaaagcc
cccaccaacg agtttctacgc atga

Reference. M81786
ACCESSION M81786
LOCUS RATTRYUODCA 2452 bp mRNA
DEFINITION Rattus norvegicus ryudocan mRNA, complete cds.
ACCESSION M81786
NID 9206822
KEYWORDS ryudocan.
SOURCE Rattus norvegicus Epithelial flat pad cDNA to mRNA.
ORGANISM Rattus norvegicus
Eukaryote; mitochondrial eukaryotes; Metazoa; Chordata;
Vertebrata; Eutheria; Rodentia; Sciurognathi; Myomorpha; Muridae;
Murine; Rattus.
REFERENCE 1 (bases 1 to 2452)
AUTHORS Kojima, T., Shwartz, M. and Rosenberg, R. D.
TITLE Molecular cloning and expression of two distinct cDNA-encoding
heparan sulfate proteoglycan core proteins from a rat endothelial
cell line
J Biol. Chem. 267, 4870-4877 (1992)

Fig. 14



15/19

Fig. 15A

MSCV



Fig. 15B

S4

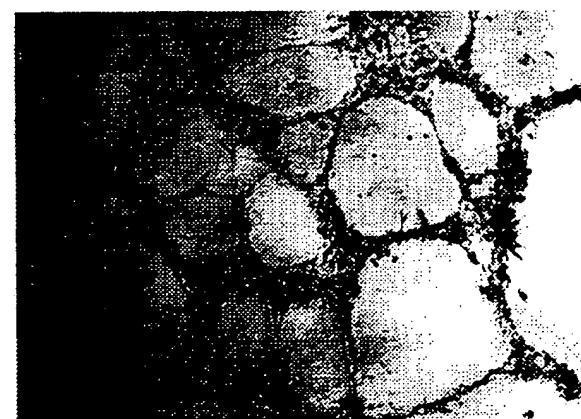


Fig. 15C

G1_{ect}-S4_{cyt}



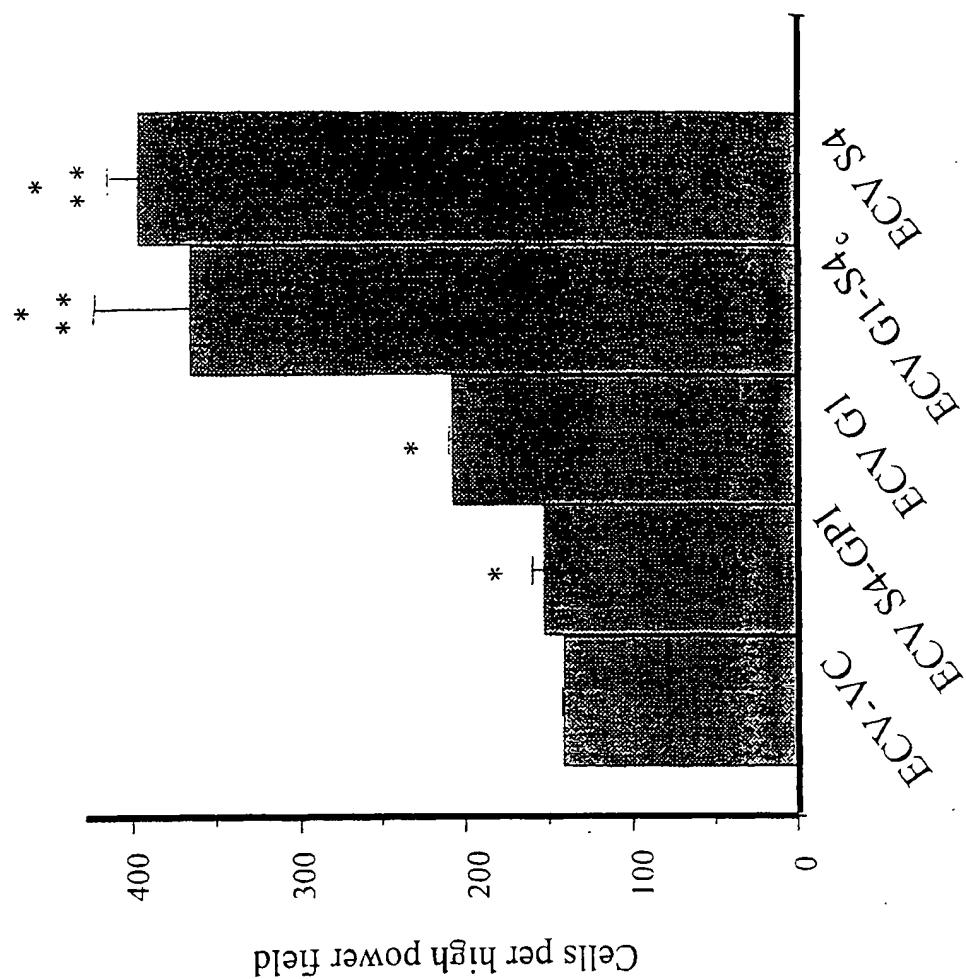
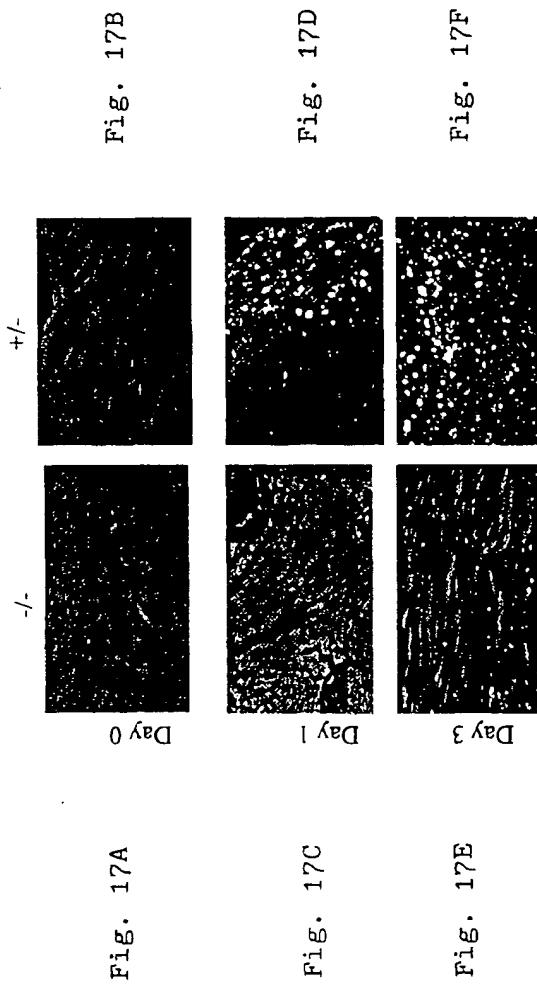


Fig. 16



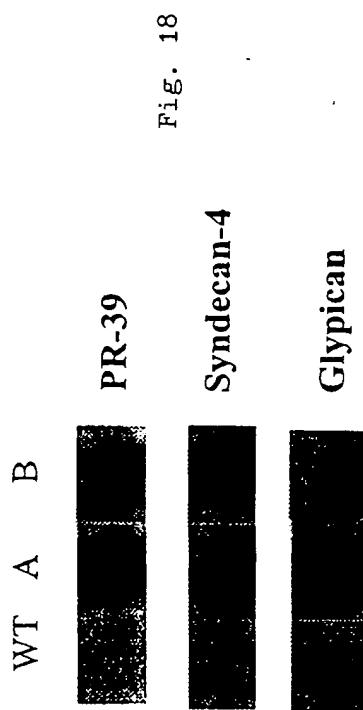
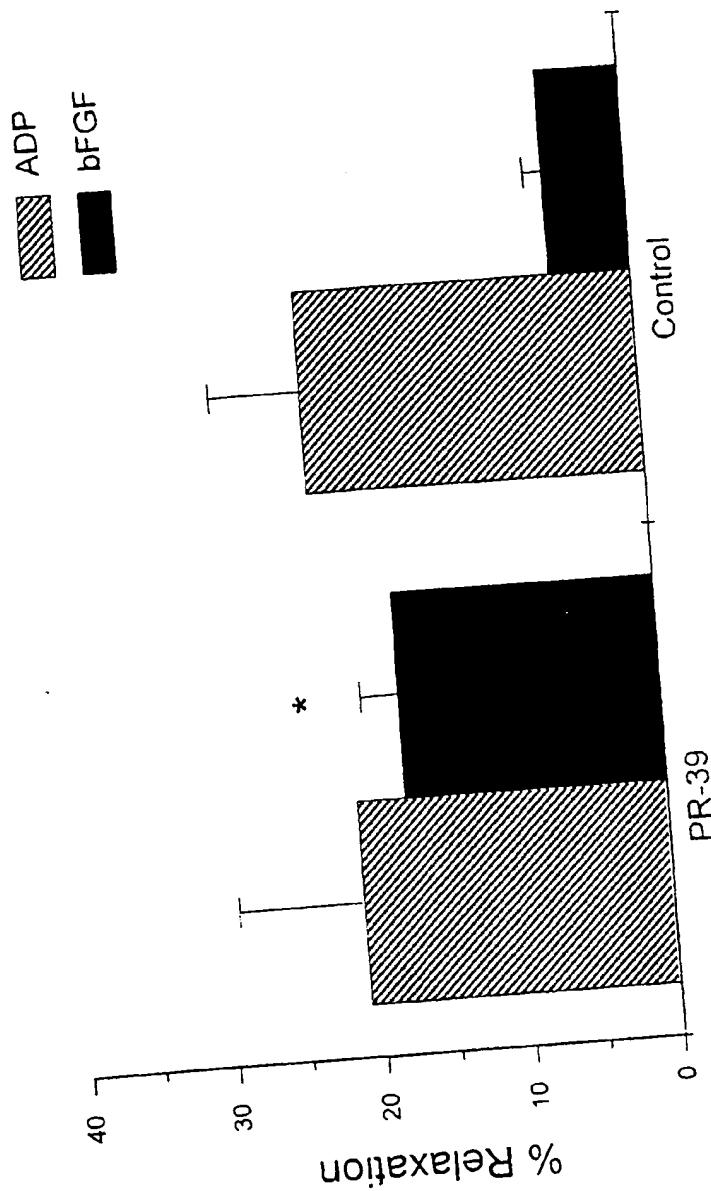


Fig. 19



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/18865

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/04; C12N 15/63, 15/85, 15/08, 15/09, 15/00; A61K 48/00;
US CL :536/23.1, 23.5; 435/320.1, 325, 440, 455; 514/44;

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 23.5, 435/320.1, 325, 440, 455, 514/44;

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BRS-WEST, CAPLUS, MEDLINE, SCISEARCH, EMBASE, BIOSIS
search terms: proteoglycan, glypican, syndecan, perlecan, extracellular domain, transmembrane domain, cytoplasmic domain

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STANLEY et al. Heparan Sulfate-Mediated Cell Aggregation: Syndecans-1 and -4 Mediate Intercellular Adhesion Following their Transfection into Human B Lymphoid Cells. Journal of Biological Chemistry. 1995. Vol. 270, No. 10, pages 5077-5083, see especially page 5077.	1-6, 10, 13
X	OKAYAMA et al. Functional Specificity of Syndecans. Trends Glycosci. Glycotechnol. 25 June 1998. Vol. 10, No. 52, pages 211-221, see especially page 213.	1, 4, 5
Y		-----
A	US 5,486,599 A (SAUNDERS et al.) 23 January 1996, abstract, cols. 2, 5, and 6.	2, 3, 6, 10, 13

Further documents are listed in the continuation of Box C. See patent family annex

* Special categories of cited documents:	*T*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L	*A*	document member of the same patent family
O		
P		

Date of the actual completion of the international search	Date of mailing of the international search report
16 NOVEMBER 1999	04 FEB 2000

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DEBORAH J. R. CLARK Telephone No. (703) 308-0196
---	---